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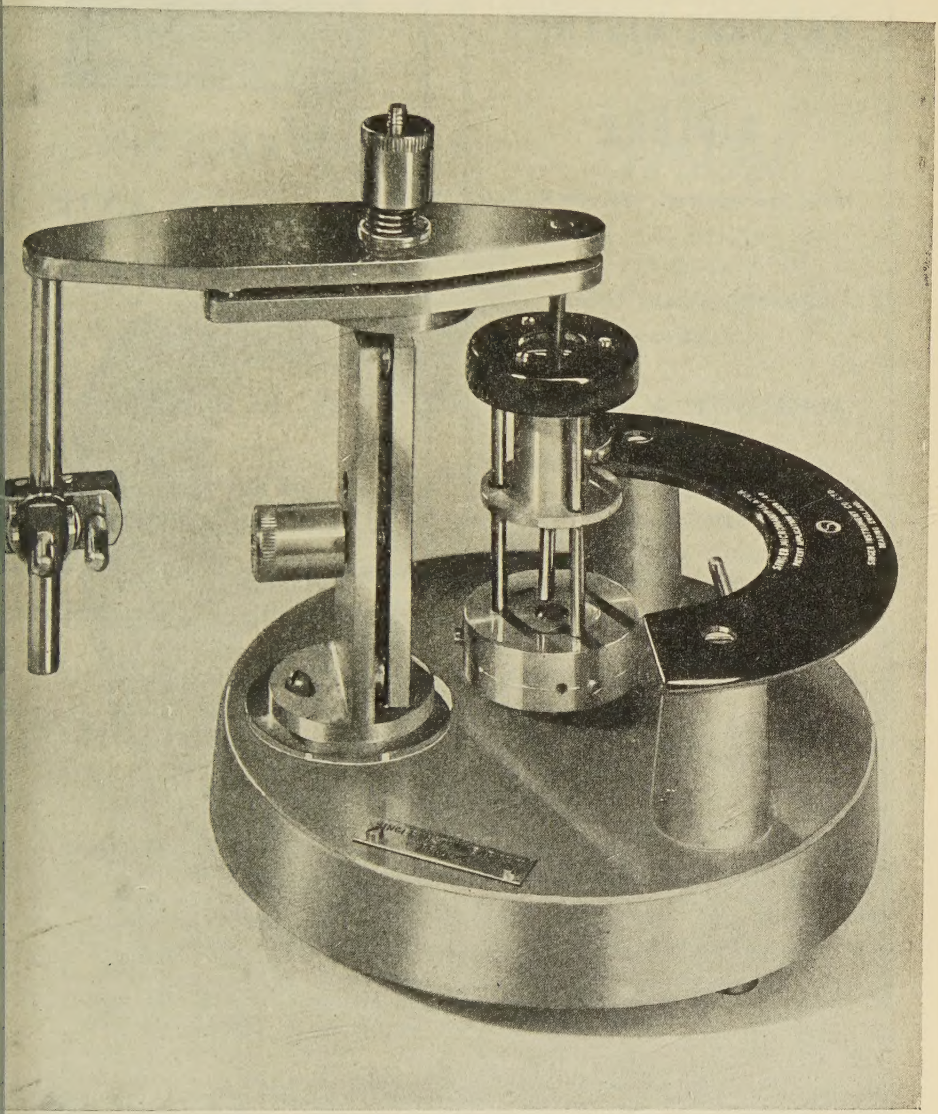

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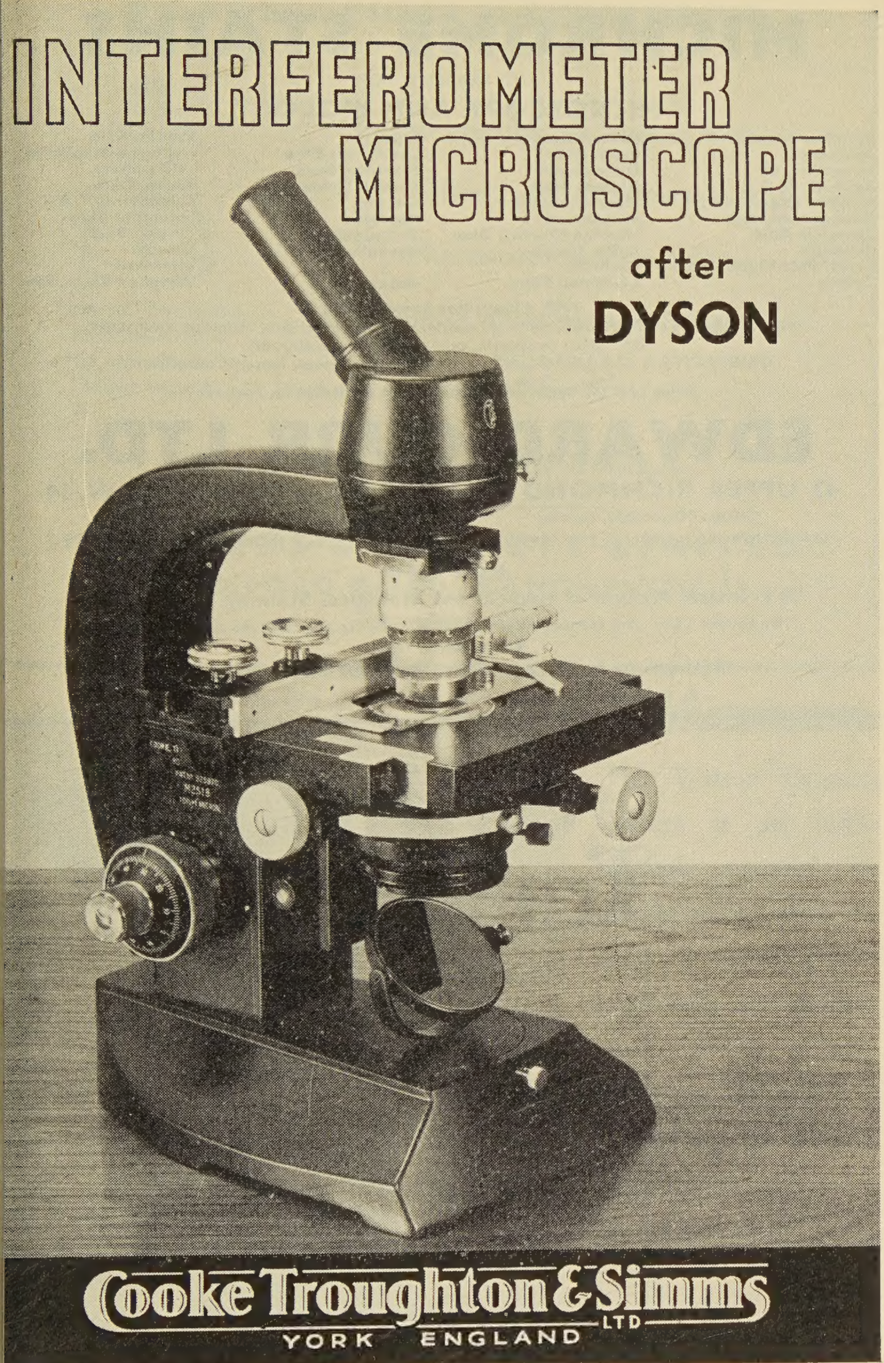

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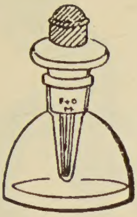

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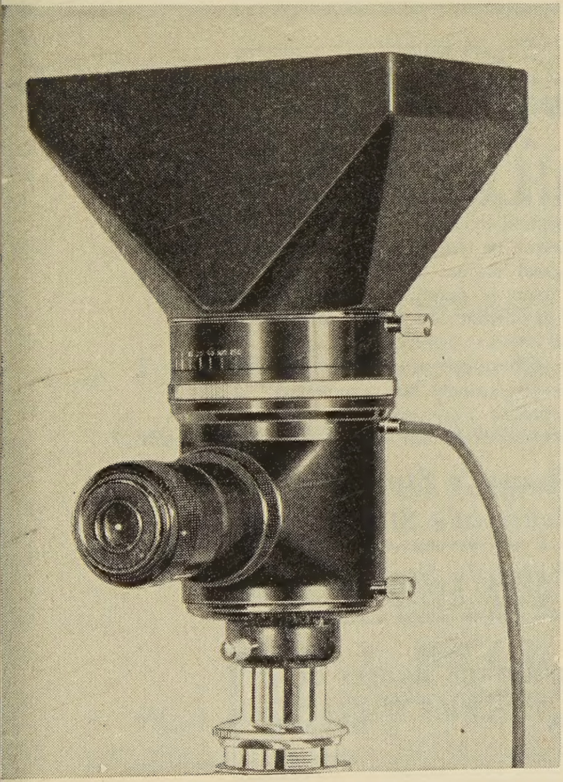

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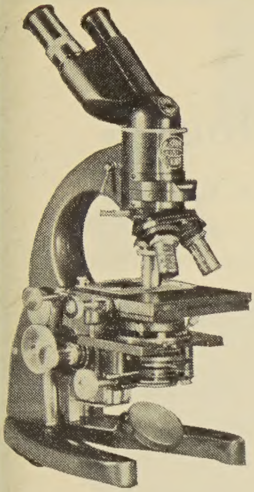

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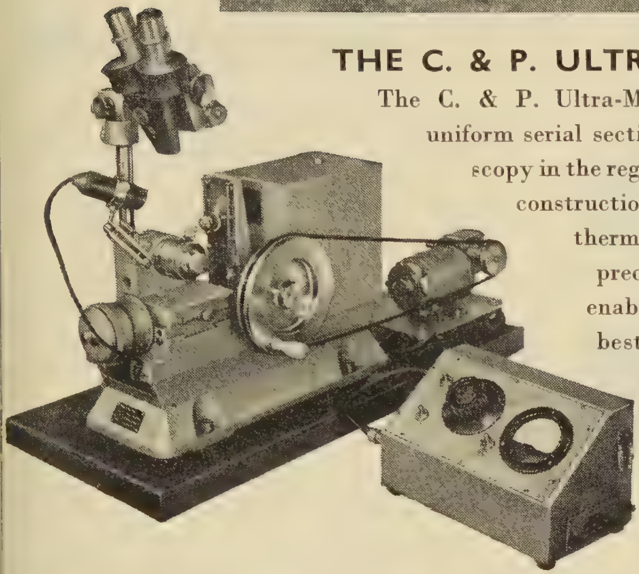

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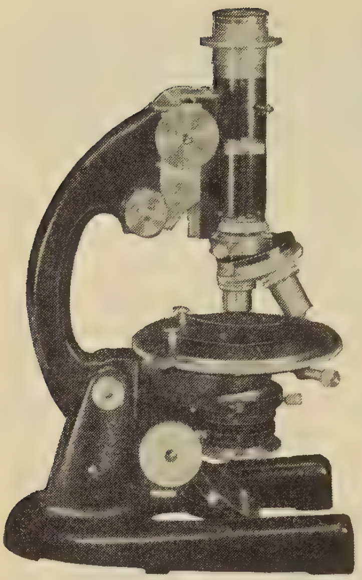

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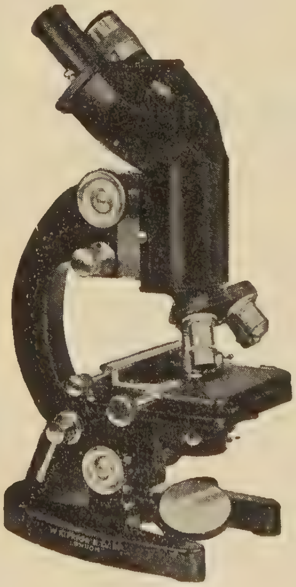

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Properties of Slime-Mould Amoebae of Significance for Aggregation

By B. M. SHAFFER

(*From the Department of Zoology, Cambridge University, and
Department of Biology, Princeton University*)

With four plates (figs. 1–4)

SUMMARY

1. When all available food has been consumed, the amoebae of *Dictyostelium* and *Polysphondylium* aggregate towards collecting *centres*; this movement involves chemotaxis.

2. Just after they have finished feeding, the amoebae adhere little or not at all to one another if they come in contact; they do not secrete the chemotactic agent acrasin, and cannot orient in an acrasin gradient.

3. Later they become able to respond to an acrasin gradient, if this is great enough. In the absence of one, they may be sufficiently adhesive to form loose amorphous associations, or, depending on the species and the environment, still be quite separate. Amoebae with similar properties may be released by the spontaneous break-up of aggregated masses. In some species, amoebae that are unaggregated may lose their sensitivity to acrasin after a few hours.

4. The elongated amoebae attracted to a source of acrasin may condense into *streams*. Stream amoebae move at varying speeds; they adhere strongly to others of the same species and to a variable extent to those of different species. They secrete the acrasin characteristic of their own species. Along a stream, there is often no continuous centrifugal decrement in secretion, as far as can be detected. The amoebae in it can be guided by the direction in which their adherent neighbours are flowing, though if they *are* exposed to an adequate acrasin gradient they will respond to it. Commonly, an adjacent stream or a centre planted beside them is not strong enough to attract them, unless they are freed to some extent from the influence of their neighbours, especially those ahead of them.

5. Except for their being effectively non-motile and more nearly isodiametric, the amoebae in a centre are similar to those in a stream and may secrete acrasin at much the same concentrations as these do. With both types, there may be short-term fluctuations in secretion, and in at least one species a long-lasting decrease.

6. The dimensions and gross appearance of an aggregation at the population densities used are partly determined by the time intervals over which the amoebae become acrasin-sensitive and centres are initiated, and by the relationship between these.

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[Quarterly Journal of Microscopical Science, Vol. 98, part 4, pp. 377–392, Dec. 1957.]

INTRODUCTION

THE amoebae of the cellular slime moulds *Dictyostelium* and *Polysphondylium* remain in the vegetative phase, dividing and moving about independently of one another, until the bacterial food supply is exhausted; they then co-operate to build stalked aerial fruiting bodies containing generally between a hundred and a hundred-thousand cells (Raper, 1940 *a*, *b*; 1941*a*). The first step in this process is *aggregation*; and at the population densities with which we are here concerned, this is brought about by a few of the randomly moving amoebae coming to rest and acting as *centres* on which the others converge, condensing as they do so into more or less radial *streams*. Bonner (1947) demonstrated that the amoebae were guided by a gradient of a chemical he named *acrasin*. This has now been isolated in stable form and its elusiveness shown to be due to its being rapidly inactivated extracellularly by a protein also secreted by the amoebae (Shaffer, 1953, 1956).

The properties of the amoebae in the various states between the growth phase and the aggregated cell mass must be described before giving an account (Shaffer, 1957 *a*, *b*) of the transformations from one state to another. As aggregation is essentially a morphogenetic movement, it is clear that the properties that are especially relevant to this process are those connected with the ability of the amoebae to guide and be guided by one another, namely, their capacity to secrete acrasin, to orient in a gradient of it, to move in a polar fashion, and to adhere when they come in contact. Earlier information on these matters comes mainly from the work of Raper (1940 *a*, *b*; 1941*a*), Raper and Thom (1941), and Bonner (1947, 1949, 1950).

MATERIAL AND METHODS

For the supply of the larger acrasin sources, slime-mould spores were inoculated with *Aerobacter aerogenes* or *Escherichia coli* on agar plates rich in nutrient, prepared according to Bonner's (1947) recipe, and allowed to develop at room temperature for 2 days. All the observations on aggregation reported in this series of papers were made on much less populous cultures. These could be most easily obtained by Singh's method (1946), with which one could ensure that when all the food had been consumed, there were still not so many amoebae present that agar could not be seen between them. The bacteria were grown alone on nutrient agar and then spread thinly on plates of 1·5% agar containing 0·5% sodium chloride adjusted to pH 6·5; the slime-mould spores were either spread with them or inoculated later in a limited area. In a few cases, which are mentioned specifically, observations were made for comparison on amoebae aggregating under water; these were prepared according to Bonner's instructions (1947).

Operations on the amoebae were carried out freehand with fine glass needles and ball-tipped rods. Except where otherwise stated, all the observations are to be taken as applying to the four species: *Dictyostelium discoideum*, *D. mucoroides*, *D. purpureum*, and *Polysphondylium violaceum*.

Observations and Experiments

ldhesiveness

Vegetative and preaggregation cells. The forces of adhesion that are of articular interest are those that bind the amoebae together. Vegetative moebae can make extensive contact with one another and then separate gain without restraint; so, in most species, can the preaggregation ones those that have stopped feeding but have not yet entered true aggregations), hough these may form temporary and everchanging clumps or join up, end o end, in short chains.

The older preaggregation amoebae of *Dictyostelium mucoroides* may form more extensive but little oriented association, to which, however, there is no ttraction from a distance; Arndt (1937) called this a *preplasmodium.* He escribed how a continuous layer of amoebae turned into an irregular and ver-changing network of tortuous strands and loops, loosely heaped up at ne nodes. Some heaps disappeared, others appeared; the mesh of the net teadily coarsened. Eventually, a few of the heaps became true centres, attract-ng other amoebae directly.

D. mucoroides includes a large number of strains, which between them roduce fruiting bodies in great variety (Raper, 1951). In the present work it as been found that the preplasmodium is prominent in one strain and not etectable in another. It has not yet been reported to occur in other species, xcept perhaps in *D. minutum*, in which some early, loose, cell associations ck any definite centres or unitary orientation (Raper, 1941*b*). Yet its presence eems to represent no great divergence; for all its various patterns can appa-ently be accounted for by the association of amoebae that crawl at random ntil they come in contact, when their mutual adhesion hinders their separa-on but is not strong enough greatly to restrict their relative movement. part from a slightly greater adhesiveness (which might well be induced in ther species by the right conditions), preplasmodial cells have the same pro-erties as other preaggregation ones.

Streams and centres. When an aggregation on agar was flooded with Bonner's 947) salt solution or tap-water, the streams and centre, not being easily etted, tended to float off on the water surface. But if they were firmly attached the agar, they were submerged; and then, with glass rods, the streams could e lifted off under water as coherent and flexible ribbons.

Streams free of the substratum, albeit very thin ones, formed spontaneously hen the amoebae were suspended in a highly viscous solution of methyl llulose. The aggregation patterns were three-dimensional; some of the reams twined loosely round one another, and the cells were cylindical rather an flattened.

The coherence of free streams and their resistance to mechanical manipula-on could not have been due to the individual movements of a collection of lls responding to a chemical gradient, even if the latter could have been own to exist; and this argument applied yet more strongly to centres. The integrity of both these stages must have been maintained in part by strong intercellular bonding. Because of the morphogenetic importance of this property, it was useful to distinguish as *integrated* and *unintegrated*, respectively, those cells that possessed a high degree of stickiness—namely those in streams and centres—and those that did not. The precise degree, though, varied with the conditions; for example, streams were much finer and broke more readily if they had formed under water rather than on agar of the same ionic composition.

The work of Raper and Thom (1941) suggested that cell adhesion during aggregation was highly specific, and this has been confirmed. During temporar immersion in water, a short section of a stream of *D. discoideum*, *D. mucoroides*, or *D. purpureum* could be placed in contact with another stream of any of these species. Allowing for the disturbance produced by this operation, it was found that the two groups of cells impeded each other's movements if their polarities were opposed. In contrast, when cells of one of these species and a centre o any of them were deposited on opposite sides of a stream of *D. minutum* o *Polysphondylium violaceum*, the cells, if sensitive to acrasin (see below), were attracted to the centre and were able to reach it by crawling right through th foreign stream, slowly forcing their way between its amoebae, a manœuvr that produced interpenetrating but non-interfering streams at right angles Cells of *Dictyostelium discoideum*, *D. mucoroides*, and *D. purpureum* wer attracted also to the older centres of *Polysphondylium violaceum* (Shaffer, 1953) both primary and secondary ones (see Shaffer, 1957*a*), and to its developin fruiting bodies (figs. 1, A, B); and they were not prevented by surface differences from entering them, or at least from applying themselves to them s intimately as not to be visible as separate groups. In contrast, though the cell of *P. violaceum* could be attracted towards a stream of *Dictyostelium minutum* on arrival they formed a separate stream alongside it; this could be taken a evidence of greater dissimilarity in surface.

FIG. 1 (plate). Photographs of living cultures. The scales represent 200 μ.

A *1*, heaps of cells taken from *Dictyostelium discoideum* streams, deposited in a row paralle to a stream and developing fruiting body of *Polysphondylium violaceum*. The stream enter the fruiting body, which is still lying flat on the agar, at the arrow. *2*, the two heaps opposit the fruiting body have moved directly towards it and in part entered it; the others have joine up into a stream and are following them.

B, cells taken from *Dictyostelium mucoroides* (*D.m.*) streams and deposited near the top o the field, left of middle, crawling through a *Polysphondylium violaceum* (*P.v.*) stream to reac and enter a developing *violaceum* fruiting body. The majority of a group of *violaceum* strean cells deposited at the same time near the top, right of middle, have already entered the mai *violaceum* stream, leaving a visible track (arrow) behind them.

C, *Dictyostelium purpureum*. *1*, aggregation in an area where the majority of cells ar effectively insensitive to acrasin. *2*, 25 min later. The streams have disintegrated almos everywhere; but their sites are still just discernible, as their cells have not yet complete dispersed.

D, *Polysphondylium violaceum*. 1, 4 centres, 1 very small (arrow), without streams. 2, 1 later. The centres have grown considerably, though still almost devoid of streams. Some the unaggregated cells have just been swept into heaps near two of the centres; they imm diately form streams (*3*).

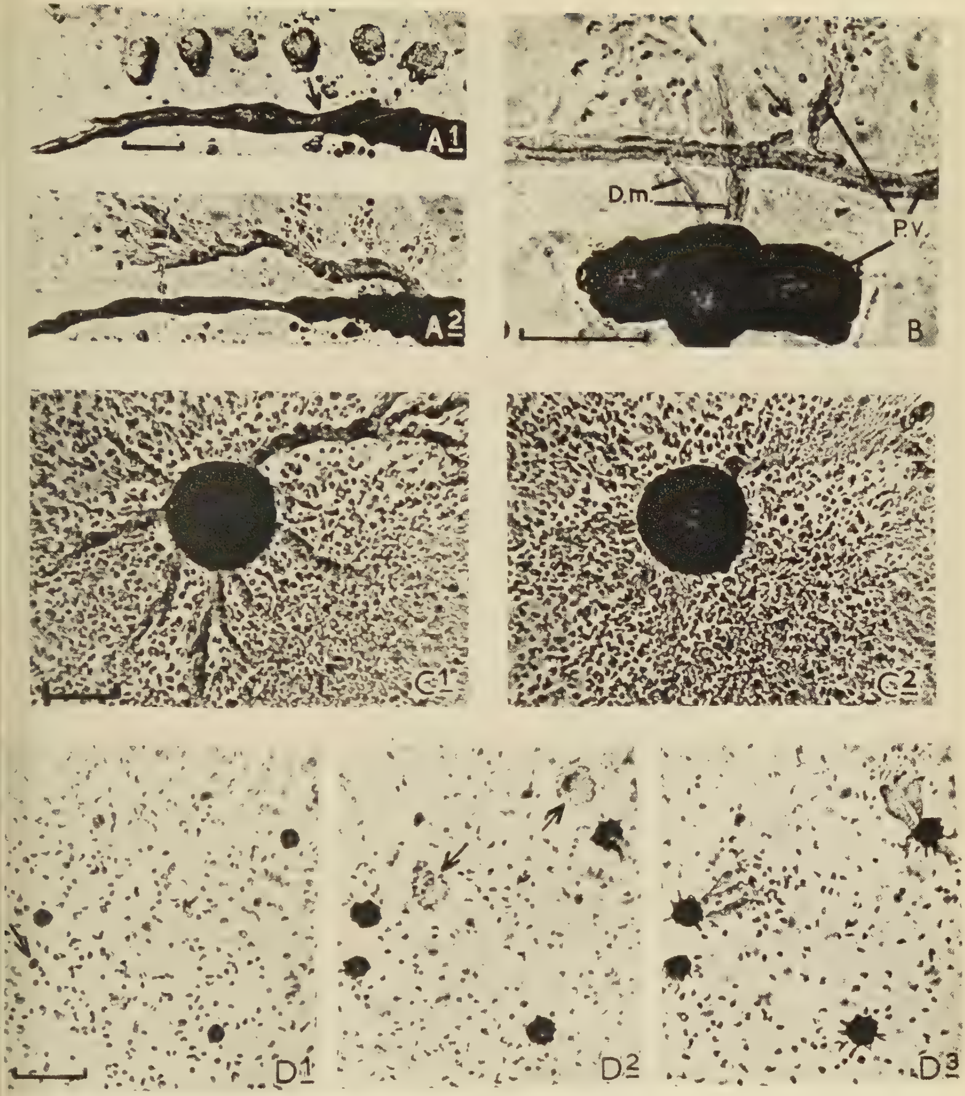

Fig. 1

B. M. SHAFFER

A sharp decrease in the intercellular cohesive forces—a return, more or less, to the level of stickiness characteristic of preaggregation stages—might occur spontaneously or might be provoked experimentally; it led to the *disintegration* of streams (fig. 1, C *2*) and centres. The degree of dispersion of the amoebae depended on the residual adhesion, which varied both with the species and the environment. The following specific differences occurred on saline agar.

In *Polysphondylium violaceum*, the part affected dissolved completely into separate cells, which wandered about in their private ways and were indistinguishable from preaggregation cells. For a short time its site could be identified by the presence there of greater numbers than on the surrounding agar; but such population differences were rapidly obliterated as the cells travelled their random paths.

In *Dictyostelium discoideum* the cells often remained initially in a more or less continuous sheet or a coarse and irregular network—a single layer, so that its width depended on the depth of the original stream. The residual adhesion did not restrict the relative movements of the cells sufficiently for them to retain the uniform orientation characteristic of the stream, nor, if reintegration did not soon occur, did it prevent their further separation. Those released from a stream were at times able to form short chains a few cells long, as could the preaggregation ones; unlike streams, these chains could crawl in opposite directions even when they were in contact.

In a strain of *D. mucoroides* possessing a preplasmodium, the residual stickiness was considerable. A compact, well-defined, and neatly ordered stream, several cell layers deep, flattened and expanded into a less refractive ribbon, much as a drop of liquid of high surface tension flattens and spreads if this tension is lowered considerably. The edges ceased to be sharp and straight and became frilled with irregular eruptions.

Differences of behaviour also occurred in a single species in different environments. Thus under water, disintegration in *D. discoideum* yielded separate cells immediately; this could be related to the poorer cohesion of an underwater stream.

Motility

Separate amoebae that were not mutually adhesive and were not subjected to an acrasin gradient crawled at random over the substratum. An analysis of time-lapse films and of the trails left on agar by single cells shows that within a population there might be wide variation in the rate of turning; and as individual cells often maintained these rates for many hours, their paths ranged from almost straight to so tortuous that there was little net displacement. When cells became sticky, their movements were modified by mutual contact; and those strongly bound in a stream had comparatively little independence, though they might flow past one another. On reaching a centre, cells ceased to be elongated and flattened and became more nearly isodiametric, a change connected with a marked loss of motility: usually, apart from small and slow changes in shape, there was almost no general movement of the mass till it began to migrate or to form a fruiting body where it was, though sometimes there might be slight shifts in position, which apparently were undirected.

Orientation to acrasin gradients

To study the acrasin secretion of compact migrating aggregates of *D. discoideum*, Bonner (1949) transplanted them into fields of amoebae that were just beginning to form aggregations and which he had found to be sensitive to acrasin gradients. Pfützner-Eckert (1950) employed a similar technique with *D. mucoroides* to investigate the properties of the separate amoebae. She used the formation of streams as a criterion of response to the aggregation stimulus; but this was not an entirely satisfactory choice, as their presence would not have revealed, without further observation, whether or not the transmission of the stimulus was dependent on cell contact, neither would their absence (nor even the absence of cell orientation) have shown that the cells were *not* reacting to the transplanted masses.

In the present work, the cells tested were exposed to acrasin sources of their own species. At first, the sources used were the migrating aggregates into which the centres of *D. discoideum* developed, which were known to secrete acrasin (Bonner, 1949), and the young developing fruiting bodies of other species, which had similar properties; these were chosen because they were protected by a slime sheath and so were easy to handle. Later, it was found that the larger and firmer of the naked centres also could be successfully transplanted. With cultures on agar, when the source was too delicate to be moved, the test cells were gathered up and deposited in heaps or streaks near it. Whether the source was transplanted or not, the response of cells in a stage when they were scattered over the agar could be more clearly defined by sweeping them together into a heap close to it and then observing their spread over the bared areas that surrounded them (fig. 1, D).

Vegetative and preaggregation cells. No vegetative cells were oriented by acrasin. The properties of the preaggregation ones varied with the species. In *D. discoideum* sensitivity to acrasin began to develop slowly throughout almost all the population as centres appeared. A few cells never did become sensitive: time-lapse films showed them wandering about at random within the aggregation area; and isolated cells were always to be seen after the main host had left the field. In *D. mucoroides* confirmation was made of Pfützner-Eckert's finding that preplasmodial cells, but not earlier preaggregation ones, would form streams leading towards an implanted centre several hours before centres would develop spontaneously; and it was further shown that these cells would orient to a source from a distance. In *D. purpureum* and *Polysphondylium violaceum* the centres might increase in size for several hours without any streams developing, or with none but the shortest of them (fig. 1, D *1*, *2*); however, this was not necessarily an indication that the unaggregated cells were insensitive to acrasin gradients (fig. 1, D *3*). Actually the proportion

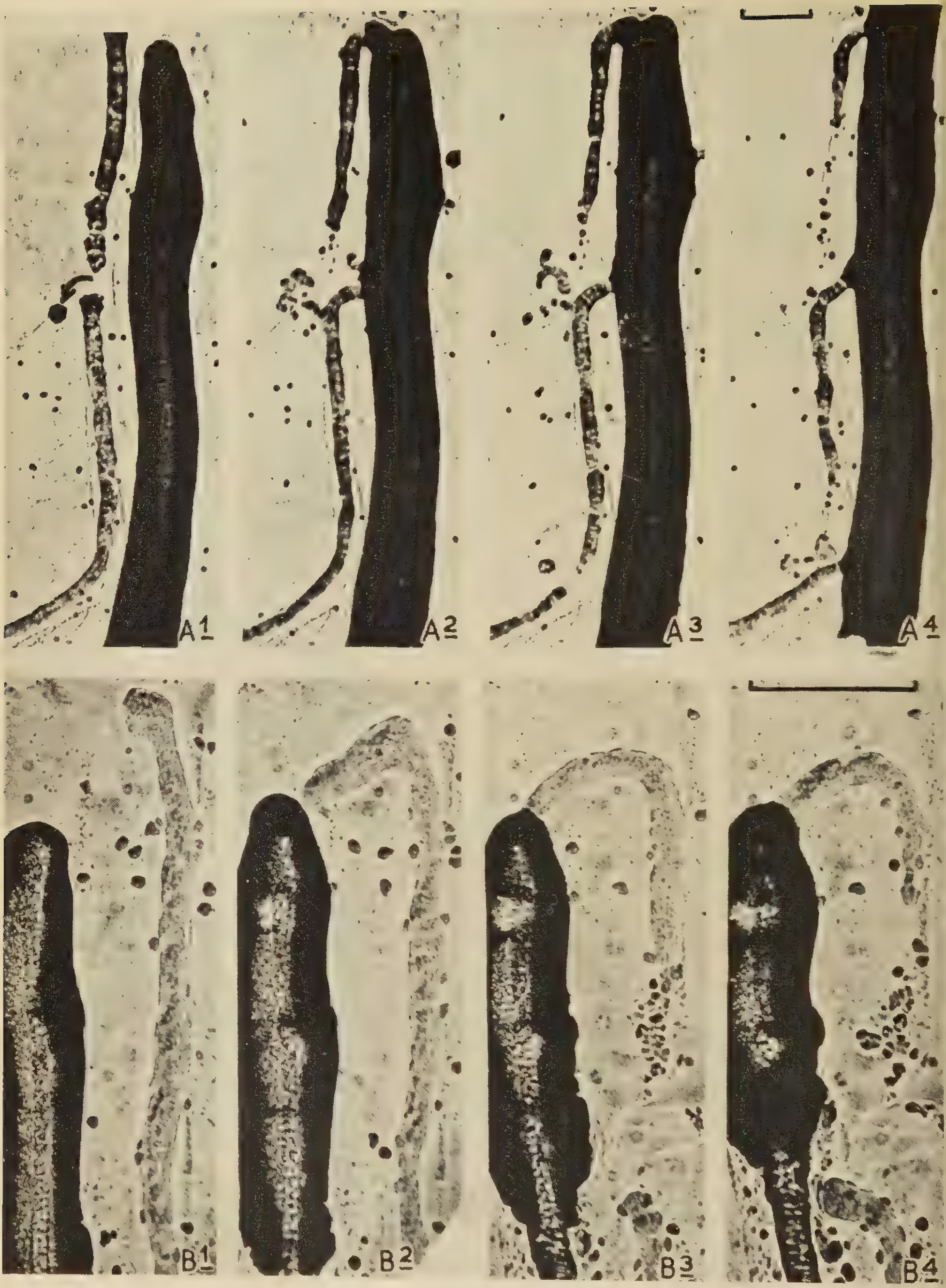

Fig. 2

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of cells that could be attracted to centres that had developed among them was very variable: when large, few cells failed to join an aggregation; when small, the streams that did form were unsubstantial and sparse and were superimposed and almost invisible on a carpet of separate cells (fig. 1, C), which was left behind after aggregation had finished. And cells that had given a strong positive reaction to temporarily implanted sources might lose their responsiveness completely after a few hours, if by then they had not been incorporated in aggregations. Their sensitivity might reappear hours or even a day later, or the cells might remain insensitive and unaggregated.

Cells released by the disintegration of a stream or centre moved towards an acrasin source, separately at first, if disintegration had proceeded as far as that, or joined in numerous chains. The degree of residual stickiness that kept cells in a nearly continuous sheet when there was no detectable acrasin gradient was not enough to prevent their escape when there was one. A small number were usually left behind: possibly they had lost their sensitivity; more probably, they had never been attracted to a distant source but had merely wandered into an aggregation. By cutting out strips of agar, the cells from disintegrated streams of *P. violaceum* could be isolated from the rest of a culture. On their agar islands, protected from the influence of any still-integrated cells, they might among themselves develop one or more primary centres, which served as nuclei for their renewed aggregation. But if such centres did not appear soon, the cells might lose their sensitivity, just as pre-aggregation ones had done, and like them might later regain it and their ability to initiate centres.

Streams and centres. If an acrasin source was carefully planted beside a compact, continuous, and smooth-edged stream, without disturbing the set of the stream cells by direct contact or the production of currents in the moisture layer on the agar surface, most commonly the cells opposite the source were not attracted to it, even if it was only 50 μ away, nor did the stream as a whole develop a curve, although separate sensitive cells at a distance of 200–300 μ would readily turn towards it. Even the thinnest stream, perhaps only a single line of cells, often remained intact. If, after half an hour or more, it was cut through opposite the source, the cut end further from the centre, if it moved at all, then did react to the lateral gradient, dragging the rest of the stream behind it (fig. 2, A). If the cut was made rather nearer the centre, the cut end had to turn back sharply to reach the source on its flank (fig. 2, B); and the section following it took the same indirect path—flowing forward too far

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FIG. 2 (plate). Photographs of living cultures. The scales represent 200 μ.

A, response of three cut sections of a *Dictyostelium mucoroides* stream to a migrating slug of *D. discoideum*, lying on the agar close by, that failed to influence the course of the intact stream. Two cuts made at the same time (*1*), one just beyond the upper edge of the field; the third, later (*3*). The cells displaced in making the middle gap, and deposited near by in a hexagonal heap (*1*), form, after de-integration (2), a short stream (3), before entering the nearest section of the main stream.

B, *D. mucoroides*. Similar to A, showing a stream cut through downstream of a fruiting body on its flank turning back sharply to reach it (*2*). Subsequently (*3*) its course becomes less angular, and it is cut through a second time.

and then making a hairpin turn. If a second cut was later made closer to the lateral source, the section behind was enabled to take a more direct route to it. Sometimes streams broke in two spontaneously, the thinnest ones doing so most frequently: the broken ends of the outer sections behaved in all things just as if they had been cut.

When a glass rod was drawn across a moderately wide stream, merely disorienting some of its cells without introducing a gap, sometimes the flow was undisturbed, sometimes the cells involved, presumably being of low inherent motility, were enabled to slow down or even come to rest. If the lesion was made opposite a transplanted source, the stream often 'breached its banks' at this point and flowed towards it. Thus, for a time, the stream forked, part leading to the original centre and part to the lateral one; but usually one arm broke or dwindled to nothing. There was a similar outcome if the edge of a stream facing a transplanted source was merely pricked, or if water currents set up on the surface of the agar in planting the source disturbed the arrangement of the cells. Most of the cells along the side of the stream in the area affected reoriented independently.

After sections of a stream had been covered by a small drop of water, they could be detached from the agar and rearranged to form a new pattern before the water had been absorbed. In this way, joining the front of one segment on to the rear of another so as to preserve the original polarity, a J-shaped stream was constructed with a centre at the end of the shorter arm. Frequently breaks and disintegrations, which were not related to the position of the centre, followed this treatment; but when the whole J remained intact, the cells in the long arm, though they might pass within 100–200 μ of the centre, were forced to make a detour of perhaps a millimetre or more before reaching it.

By exploiting the positive phototaxis of a migrating aggregate, or 'slug', of *Dictyostelium discoideum* (Raper, 1940*b*), it was possible to bring this acrasin source into much closer contact with an undisturbed stream than could be achieved by transplantation alone: by appropriate illumination it could be induced to creep over a *discoideum* stream. Of this, all or part of the section upstream of the crossing (but not of the section nearer the centre) did sometimes follow the slug, a fate that could to an extent be correlated with the degree of mechanical disturbance produced; but frequently the whole stream was left intact (fig. 3, A), thus witnessing the impotence of this source even at close range. One complicating factor eliminated by the presence of the slug's sheath was the possibility of adhesion between the two bodies. That this membrane was an effective barrier was shown most clearly when a slug had begun to migrate while one or more streams were still entering it. These flowed after it, travelling inside the slime sheath, which a slug left behind itself, collapsed, as a slime trail; and thus enveloped, they could be led over a naked stream without disturbing it (fig. 3, B). It was possible to cross one *naked* stream over another one while they were temporarily submersed in water; these, however, invariably stuck together and interfered with each other.

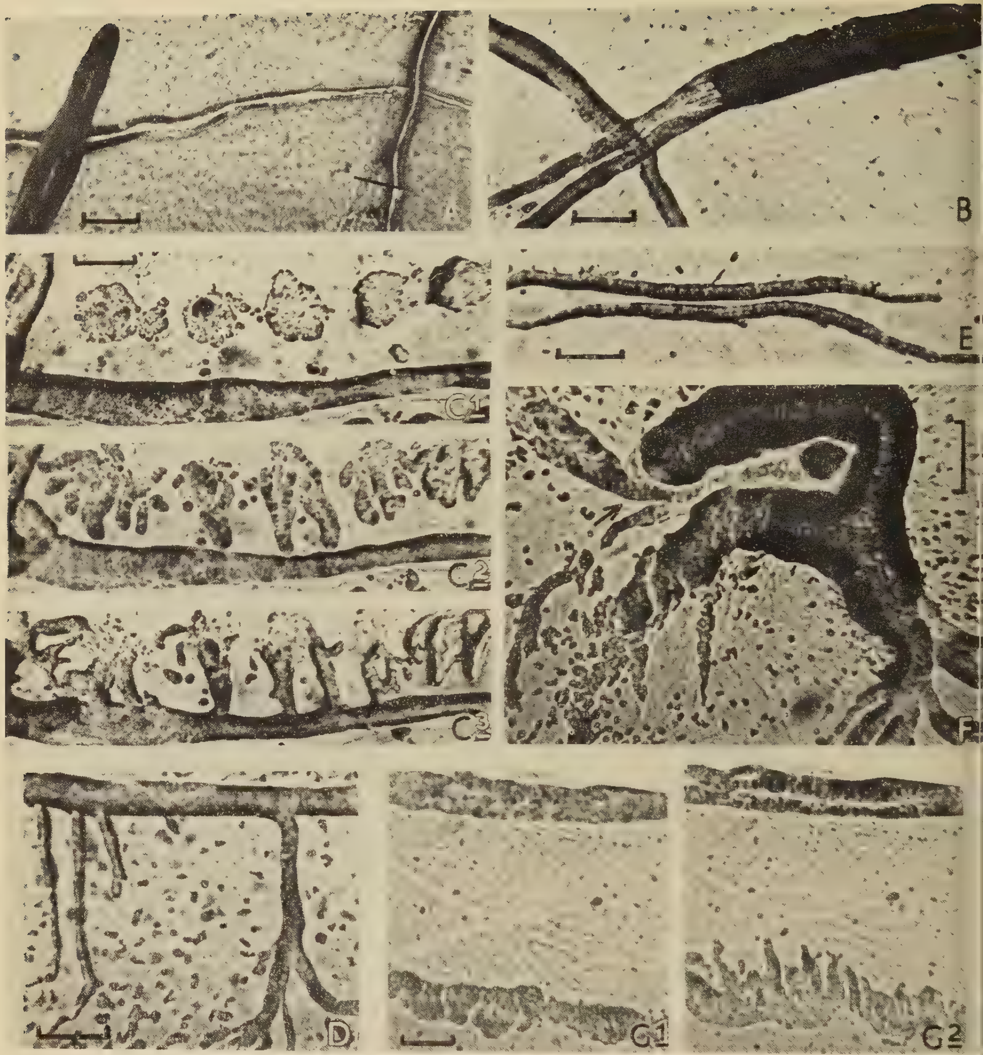

FIG. 3

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Certainly, closely knit streams sometimes *were* affected by a lateral source. Both spontaneous disintegration and transverse breakage were liable to occur independently of the presence of such a source, and the cells thereby released were free to react to one if they were within range of it. But additionally some responses were apparently not preceded by either of these phenomena, though usually in such cases few cells reoriented independently and the rest just followed them. However, what demanded attention was not that a lateral source might attract stream cells but that it commonly did not do so.

That a single line of stream cells in contact only at their tips might not respond to a transverse acrasin gradient, though all their sides were exposed to the external medium, indicated that even with this stimulus they could produce pseudopodia only at their front ends and not from their sides. This conclusion together with the experiments just described tended to show that the cells in a compact stream would in general respond to a natural acrasin source external to the stream only when their front ends were freed to some extent from the influence of their neighbours. A study of the behaviour of the outer end of a stream and of the cells central to a cut showed that exposure of the back end of a cell did not make it more sensitive to an external source.

Streams formed under water reacted in much the same way to lateral acrasin sources as did those on agar, though their cells were attracted more frequently to sources planted very near by; but then, under water, the properties of the cells were slightly different, as was evident from the fineness and diffuseness of the streams.

A *centre* typically did not move in an external acrasin gradient: two centres might form or be planted very close to each other and yet not draw together.

Acrasin secretion

Having studied sensitivity, it was possible to select cells for the detection of acrasin gradients. Of the preaggregation cells, only the preplasmodial ones of *D. mucoroides* were consistently sensitive; cells from recently disintegrated streams of all species were suitable but were not always available in sufficient quantity. The streams themselves were clearly an immense store of potentially sensitive cells. If a fine glass rod was dipped into one of them and then allowed

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FIG. 3 (plate). Photographs of living cultures. The scales represent 200 μ.

A, *Dictyostelium discoideum.* A stream crossed on the left by a migrating slug, and on the right by a stream flowing inside the slime sheath deposited by another slug (width indicated by ink line), which crossed over earlier.

B, *D. discoideum.* A stream crossed by a slug and by the pair of streams following it inside its sheath.

C, *D. mucoroides.* Acrasin-sensitive cells, deposited in a row of heaps beside a stream, used to test for a differential in acrasin emission along it. The centre and another stream are at the eft.

D, *D. mucoroides.* Groups of acrasin-sensitive cells, attracted to a stream, oriented perpendicularly to it.

E, *D. discoideum.* Two long-established streams separated by barely a cell's width.

F, *D. discoideum.* A thin stream flowing independently between two massive streams, hough almost in contact with them.

G, *D. mucoroides.* *1*, two parallel and relatively well-separated streams, one of them stirred p. *2*, 30 min later. The cells in this one beginning to respond to the intact one.

to touch an area of bare agar, it carried over some of the cells and deposited them in a small heap, at the same time destroying their intercellular organization. For a time they stayed rounded up and inactive; then the heap developed in one of the following ways: (1) it did not move even when exposed to an external acrasin gradient; (2) it moved only if so exposed; (3) it moved without needing to be stimulated by a gradient; (4) it disintegrated. Non-motile cells were unable to give information about acrasin gradients, but virtually all cells that moved (developments 2, 3, 4) were sensitive, and these were most surely obtained by rapid transfer from flat streams on moist agar. They were deposited in heaps at various distances up to a few hundred microns from the object to be tested.

Vegetative and preaggregation cells. These did not secrete acrasin, as far as could be detected by the indicator cells. (Sensitive preaggregation cells, including those in short chains and in the preplasmodium of *D. mucoroides*, could scarcely have done so and not formed true aggregations.)

Streams and centres. In general, the indicator cells crawled directly towards centres, slugs, and developing fruiting bodies of their own species, whether the cells were planted near these masses or these near the cells; it was, however, almost impossible to pick up young centres, and slightly older ones were often so damaged in transit that, at least temporarily, they ceased to be attractive. The old centres and young fruiting bodies of *Polysphondylium violaceum* were exceptional in that often they attracted cells of their own species poorly, while they did attract those of many *Dictyostelia* (fig. 1, A, B; Shaffer 1953).

From the fact that cells formed streams instead of flowing directly towards the centres, Bonner (1949) concluded that the streams produced acrasin also, though to a lesser extent. An equally cogent argument for the emission of acrasin by peripheral cells is that the greatest distance they can be directly attracted by a centre is about 350 μ (Bonner, 1947), whereas an aggregation may extend for centimetres. Bonner (1949) was able to investigate the differential emission along a slug by recording the alignment of all the amoebae reacting to it, as this indicated the maximum gradient where they were. This method has been applied to emission by a compact stream by laying heaps of sensitive cells beside it at intervals for a good fraction of its length. Frequently they moved roughly perpendicularly towards the nearest part of it (fig. 3, C, D). Had there been a sustained gradient of emission along it great enough for them to detect, they would have approached it obliquely, biased towards the centre.

Though cells of *Polysphondylium violaceum* and *P. pallidum* might be attracted to a stream of *Dictyostelium minutum*, differences in surface properties prevented them from coalescing with it, so that their behaviour on reaching it was of great interest. If they continued to move, they formed a stream of their own alongside it. This stream sometimes flowed the same way as the *minutum* one, but (unless the cells had arrived at a point within direct range of the *minutum* centre) it flowed in the opposite direction just as often. Apparently the longitudinal gradient, if any, had not been steep enough to stimulate the

Polysphondylium cells asymmetrically on their arrival, and they had either chanced to turn to the left or to the right or continued straight on. Those that turned must then have been exposed to a transverse acrasin gradient and so have been prevented from escaping from the side of the primary stream, though unable to fuse with it: those that crossed it must have begun to travel down an acrasin gradient as they started to leave its far side and so have been drawn back again once they deviated in either direction from the perpendicular. The majority orientation of the earliest arrivals must have been communicated to the later ones, presumably by contact.

Further evidence that along the length of a stream there need be no persistent quantitative or qualitative differences controlling movement was obtained by cutting up a stream lying on agar into a number of sections and recombining them as desired, while they were temporarily submerged in a drop of water. The reconstructed stream, no longer attached to the centre, developed in one of a variety of ways, which are considered fully in the following paper; these were the same whether the sections were rejoined in their original order or in any other, provided only that none was reversed. The fact of immediate relevance is that sometimes all the cells continued to flow forwards till they had piled up at the new leading end.

A disintegrated segment of a stream did not secrete; nor, effectively, did stream cells that had been sufficiently disturbed but were still integrated. Thus heaps of stream cells deposited on agar not only did not move for a few minutes afterwards but did not attract sensitive cells; some later resumed secretion. The possible variation in the immediate development of such heaps is tabulated below.

TABLE

Development type	*Movement without acrasin gradient*	*Movement up acrasin gradient*	*Resumed acrasin secretion*	*Adhesiveness*
1	−	−	+	+++
2	−	+	+	+++
3	−	+	+	+++
4	+	+	−	±

In development 4, the initial disorientation was followed by disintegration; in 2 and 3, sensitivity was usually recovered before secretion was resumed. As neighbouring heaps could develop differently, a variety of interactions occurred between them.

Once it was recognized that a stream emitted acrasin, and, moreover, that it might do so without any sustained gradation being demonstrable along its length, a study of natural aggregation led to the same conclusions about stream sensitivity as had been arrived at by experiment. Streams often ran close alongside one other—perhaps a single line of cells next to a massive ribbon—without drawing together as the hours passed (figs. 3, E, F). Cells merely followed the path of those in front of them with fair accuracy; consequently, if the stream was curved, they might be led away from the centre they ultimately reached. Over a long period, the arms of a U-shaped bend in a stream, though they must have set up a gradient across each other, tended, if anything, to diverge rather than to approach. Streams, of course, did join up as they converged on the centre, but for the most part these junctions appeared when the streams first formed. A new junction might be established after limited spontaneous disintegration or transverse breakage; whether it was, depended on the reactivity of the cells leading the separated length of stream and on the direction of the maximum resultant gradient set up by all the acrasin-secreters around them. Additionally, many an intact stream locally shifted its course a little, apparently because cells followed their leaders imperfectly, so that neighbouring streams might become confluent for short distances. This behaviour contrasted with what happened when one of two streams that had been flowing parallel to each other for some time was stirred with a glass rod without greatly altering its external form: the cells along the *whole* of the section disturbed began to crawl towards the other stream (fig. 3, G), if in range of it.

One more experimental distortion of an aggregation will be discussed as it well illustrates the fundamentals of stream acrasin sensitivity and secretion. Two long streams were selected that had coursed beside each other for several hours without approaching and were free or freed from tributaries (fig. 4, A*1*). By sweeping away some of their cells, a number of gaps were made in both of them, each about as wide as the distance between the streams and separating intact sections several times as long (fig. 4, A*2*). The gaps in one stream alternated with those in the other. On occasion, there was no disintegration and the cells leading each of the sections turned and flowed towards the middle of the nearest section of the other stream. The precise pattern that resulted when they arrived there depended on the exact configuration of the streams before cutting. If they had been straight and parallel, it resembled a ladder with incomplete sides. In this case, as the cells ahead of each gap continued to move in their original direction, the pattern changed into a square-wave and finally, as the cells cut the corners, into a sine-wave. Thereafter, they were constrained to crawl along this winding course on their way to the centre. That the initial turn of the front of a section was in response to the resultant

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FIG. 4 (plate). Photographs of living cultures. The scales represent 200 μ.

A, *Dictyostelium discoideum.* The mutual attraction of the sections cut from two streams (*2*) produces a sinusoidal stream (though this soon parts at a point just ahead of a secondary centre that forms on it) (*3*).

B, C, *D. mucoroides.* The orientation of sensitive cells used as an indication of the relative acrasin emission of streams and primary centres.

D, *D. discoideum.* Response of sensitive cells to a section of stream containing an incipient secondary centre.

E, F, *D. mucoroides.* The partition of groups of sensitive cells, placed midway between streams and young fruiting bodies, used to estimate the relative emission by these acrasin sources.

G, *D. discoideum.* Similar to E. Comparison of the acrasin emission of a centre and a stream.

H, *D. discoideum.* Comparison of the acrasin emission of a slug and of a stream that in places is only a double line of cells.

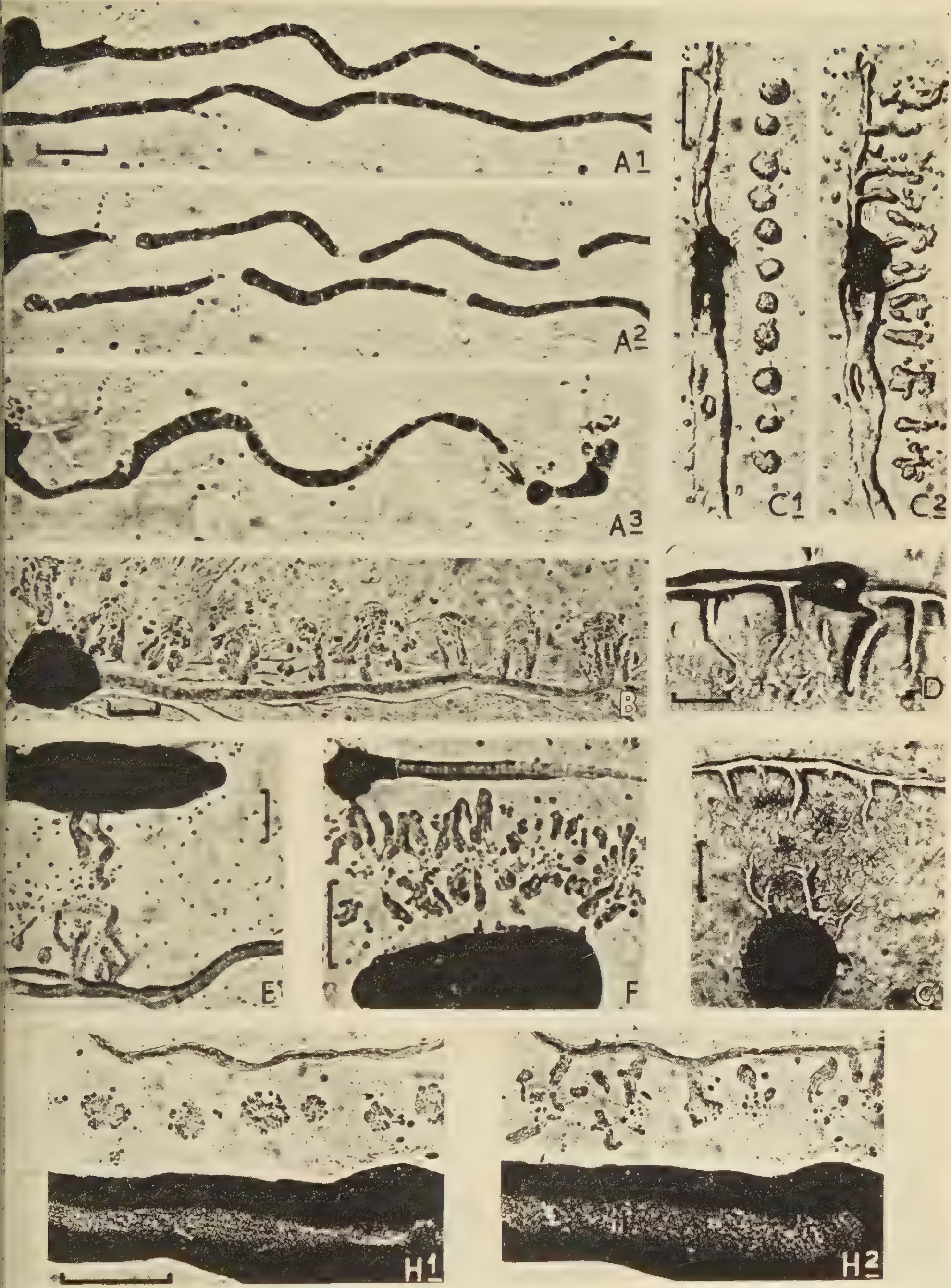

FIG. 4

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gradient is perhaps made more obvious by considering the front to have been exposed separately to the acrasin produced by each of the three sources: the part of its own stream ahead of it, the part behind it, and the other stream, only the last stimulating it asymmetrically.

The method used for studying the acrasin emission of a stream could be extended to compare this with that of a centre. Nearly half of a radial aggregation was swept away, leaving the centre undisturbed on the exposed diameter, or a centre was selected that was fed by two streams 180° apart. Sensitive cells were then piled in small heaps in a row parallel to one of these line sources and a short distance from it. In many cases, they moved towards it approximately along the normal to the nearest part of its surface, even in the central area (fig. 4, B, C). A similar result was obtained when the line source was a stream still flowing into an early migrating slug or into a young and as yet horizontal fruiting body, or a stream on which had formed a secondary centre (fig. 4, D).

Bonner's (1949) second method of comparing the acrasin secretion of two sources was to measure the partition of a field of sensitive cells between them. Though this method has many limitations, it suffices for a rough estimation. Ideally the sources should be of the same shape and similarly oriented, time should be allowed for recovery from transplantation, and no cells should intervene between each source and test cells placed in the immediate vicinity of the zone where the gradient is effectively zero. In practice, a centre, slug, or fruiting body was laid on agar at a variable distance from a stream and, if elongated, parallel to it; and about 15 min later, sensitive cells were deposited in a streak or in a single row of heaps between them, parallel with their nearer surfaces. The position of the zero gradient line was revealed only when it happened to run through the band of cells; this it often did, even if the stream was a very thin one, when the band was midway between the sources (fig. 4, E–G). In such cases it could be concluded that they were secreting acrasin at about the same concentration, despite the great difference in their size. Sometimes, however, the stream proved to be a stronger attractant (fig. 4, H).

Yet acrasin secretion was not maintained at a constant level. Fluctuations in it were probably responsible for the rapid realignments of a field of sensitive cells, revealed most clearly in time-lapse films (Arndt, 1937; Bonner's film, 1944), and for those cases where cells in an intact stream began to respond to an external source; the possibility that they also caused rhythmic aggregation movements is discussed in another paper (Shaffer, 1957*c*). Sometimes centres became temporarily unable to orient sensitive cells, even at very close range. At least in *P. violaceum*, in circumstances that have not been defined, this condition might involve whole radiate aggregations, be long-lasting, and be associated with a reduction in sensitivity of the unintegrated cells. In this event, no further cells were attracted, though the shrivelling streams flowed on into the centres. Occasionally, after a lapse of several hours, the same centres began to collect unaggregated cells again, though most of these might arrive too late to be incorporated in the fruiting bodies into which the old centres transformed themselves, in which case they were left clustered around the bases of the stalks.

DISCUSSION

In earlier studies on sensitivity to the aggregation stimulus, Raper (1940*a*) showed that preaggregation amoebae of *Dictyostelium discoideum* would not respond to implanted centres, and Pfützner-Eckert (1950) that those in the preplasmodium of *D. mucoroides* would do so. Bonner (1947) reported that when a *D. discoideum* centre was removed and placed at the side of a stream that had been flowing into it, the stream cells individually reoriented towards it. This result might well have been due to the use of two stimuli, of which the first—separating the stream from its centre—is known (Shaffer, 1957*a*) sometimes to induce the stream to disintegrate and so give its cells their independence. In contrast, there can have been no disintegration when Pfützner-Eckert (1950) laterally displaced the centre of an aggregation of *D. mucoroides* and found that the broken ends of the streams turned and flowed towards it. She also claimed that if the centre was pushed over the agar in a curve or zigzag, the streams did not orient directly towards it in its new position but followed its path. I have not been able to confirm this.

Clearly, for aggregation to occur, the development both of secreting centres and of sensitivity to acrasin is necessary. The time intervals over which these two developmental processes stretch, as well as their relationship to each other, vary considerably in different species, and even in the same species on different occasions. This in itself accounts for much diversity in the size and appearance of the aggregations.

When near an acrasin source, a separate sensitive cell can align itself with the maximum gradient in the external medium by a differential outflow of cytoplasm at its front end, which is stimulated, presumably, by the differences in concentration this part of its surface experiences; and as a secreting cell can still orient in this way, it may well be that acrasin applied to the outside of the surface has an action different from that on the inside.

Are the amoebae guided by acrasin when they are in the streams? By depositing sensitive cells beside an ordinary compact stream, one can show that there may be along it no continuous centripetal increase in the acrasin escaping from it; and the results of planting a centre near a stream, or of leading a migrating aggregate over one, demonstrate that, at least on agar, these sources may not constantly or even intermittently release acrasin at a much higher concentration than any part of the stream, including its outermost reach, ever does. Furthermore, Bonner (1950) concluded from the fact that the amoebae sometimes crawled round in a ring that a gradient was not always necessary for orienting them, because the concentration could not have gone on increasing all the way round it. But none of this evidence bears on whether there are temporary local gradients, perhaps developing in some sequence.

It is probable that the rhythmic aggregation movements reported by Arndt (1937) are associated with zones of increased acrasin secretion spreading out from the centre. These might, while approaching the stream cells, expose them to gradients great enough to cause orientation. The increased secretion might be sustained; but there is no evidence that the older centres constantly release acrasin at a higher concentration than do the younger ones. If secretion fell off again, there would be a reversed gradient following each pulse, but this would not necessarily cancel out the effect of the gradient preceding it: secretion might decrease either so slowly or so rapidly that the reversed gradient would be, respectively, undetectable or effective only for a considerably shorter time; the cells might be less responsive because of adaptation or fatigue; and in any case, an exactly reversed gradient should not cause cells that have just been aligned to change direction, if they turn in response to asymmetrical stimulation of their front ends.

Though pulses of acrasin secretion moving centrifugally probably do at times play a part in maintaining the orientation of stream cells, there is some evidence that they are not essential. Rhythmic movements are not always to be seen in films of aggregation (Bonner's film, 1944; my own), though, admittedly, this does not prove that acrasin pulses are not present. There is also the observation that segments of an isolated stream can flow forward in whatever order they are rejoined: if pulses were responsible, the pacemaker for them would have to arise at the new front end and not at the original one.

The results presented support the hypotheses that the separate amoebae are first oriented by differences in the external acrasin concentration; that after they have condensed into a stream they can be adequately guided by the general direction of flow of their fellows, for whose surfaces they have a high affinity; but that if they do then experience a large enough gradient, either sustained or short-lived, they can orient by it. Certainly the cells at the edge of a stream must be exposed to a *transverse* gradient as a result of its own secretion, and this together with their adhesiveness will prevent them from escaping. Likewise, in a stream that is only a single lines of cells, all of them will be marginal and will be oriented by acrasin, in that this will continuously restrain them from flowing out sideways. The validity of the general conclusion is perhaps attested most clearly by the behaviour of cells that have been attracted to a *D. minutum* stream with which they will not coalesce: they form a stream of their own, which can flow either in the same direction as the *minutum* stream or in the opposite one, but (in the absence of competing acrasin sources) does not leave its side. (However, the pattern of secretion in *D. minutum* may not be quite the same as in the other species considered.)

Except where it forms localized swellings, a compact stream tends to be of uniform thickness, or at least of smooth taper, between the points at which adjacent tributaries flow into it. Even when additional cell groups of various sizes reach it at different places, they are usually fitted into it without disturbing the regularity of its outline. That the number of cells lying side by side does not fluctuate considerably along a stream section can be accounted for by the combined effect of mechanical guidance and the transverse acrasin gradient.

An important role is thus ascribed to intercellular adhesiveness, as might have been anticipated from the classical work on the part it takes in the morphogenesis of other species (e.g. Wilson, 1911; Galtsoff, 1925; Holtfreter, 1944); and indeed Bonner (1950) has already shown that it is a factor in aggregation by the observation that a section of a stream reversed and grafted on to the end of another stream can be pulled 'backwards' into the centre.

Raper and Thom (1941) found that mixed amoebae of *D. discoideum* and *D. purpureum* could enter the same aggregations but produced separate fruiting bodies; whereas those of one of these species and *Polysphondylium violaceum* would not enter common aggregations but formed separate ones, with their streams overlaid. This behaviour is partly due to the use of a common acrasin stimulus for chemotaxis in the former case and different ones in the latter (Shaffer, 1953), but it must further be supposed that these *Dictyostelia* sort themselves out in streams and centres on the basis of selective affinities. I have observed the following patterns of behaviour, which are based on a gradation in contact affinities: stream cells of these *Dictyostelia* hinder one another from moving in different directions when in contact, a corollary of the guidance mechanism; they can form interpenetrating streams with the cells of *Polysphondylium violaceum* when attracted to different places, though when moving to the same place they are sufficiently alike to be apposed to them; the cells of *Dictyostelium minutum* and *Polysphondylium violaceum* build separate streams side by side even when travelling in the same direction.

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Variability of Behaviour of Aggregating Cellular Slime Moulds

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With four plates (figs. 1, 2, 4 and 5)

SUMMARY

1. A description is given of the development of certain structures—centre, stream, and ring—formed by the amoebae of four species of *Dictyostelium* and *Polysphondylium* during aggregation, and of the outcome of experimentally cutting a stream, recombining lengths of stream in simple geometrical relationships, and excising a centre.

2. In all cases there is a high degree of intraspecific variation in behaviour, and it is not possible to predict, except in terms of rough probability, what the future course of development will be.

3. An amoeba at this stage of the life cycle can exist in one of four main states. In the first, it is unaggregated and cannot react chemotactically; in the second, it is unaggregated but can react; in the third, it is in a stream moving towards a centre or has properties similar to those of a stream cell; and in the fourth, it is in a centre and nearly stationary. Though an enormous variety of patterns may be produced by large numbers of interacting amoebae, their behavioural repertory may be considered, without too much simplification, to be restricted to approaching these four states in various sequences and at various rates.

4. Most of these sequences can be observed over a wide range of environmental conditions. Changes in humidity produce the greatest effect: the drier the culture, the stronger the drift from the first state to the fourth.

5. It is suggested that variability is occasioned by the simplicity of the mechanisms controlling development.

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INTRODUCTION

THE social phase of the cellular slime moulds *Dictyostelium* and *Polysphondylium* begins some time after the available food has been consumed: the amoebae cease to live and move independently, and instead a few of them initiate collecting *centres* towards which the rest are attracted in more or less radially arranged *streams* (Raper, 1940 *a*, *b*, 1941). Specific chemotaxis is one

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of the factors involved (Bonner, 1947; Shaffer, 1953, 1956). In the first paper of this series (Shaffer, 1957*a*), the cells taking part in this aggregation were described in terms of their motility, specific adhesiveness, ability to secrete the chemotactic agent *acrasin*, and ability to orient to a gradient of it. Having characterized the performers, we may now consider the performance. In the past, perhaps insufficient emphasis has been given to the variability of behaviour of aggregating amoebae, whether undisturbed or subject to experimental interference. Thus, for example, the cutting of a stream, one of the few operations executed by several independent workers, was reported by each of them to lead to a single type of response. It was, however, of a different type in each case. This might have been due to the use of different species and culture conditions; but in the present study it has been found that all the responses they obtained, and more, can be seen in any one of a number of species over a considerable environmental range.

The object of this work has not been to compare the aggregation patterns of all known species and mutants, but rather to record the variation to be found within a species. Four species have been examined to determine whether such variability is a general characteristic: *Dictyostelium discoideum*, *D. mucoroides*, *D. purpureum*, and *Polysphondylium violaceum*. At the population densities used (at which the cells before aggregation do not cover the agar in a continuous sheet), they all commonly produce approximately radiate aggregations, which may be indistinguishable (Raper, 1940*a*); and it has been found that the range, though not the frequency, of variation is very nearly the same in all of them.

Culture methods and techniques have already been described (Shaffer, 1957*a*). Aggregation took place on agar usually containing 0·5% NaCl adjusted to pH 6·5 (Singh, 1946) but from which this salt was sometimes omitted or replaced by buffer. Occasionally, by Bonner's (1947) method, the amoebae were made to aggregate on glass under water. The following simplified and schematic descriptions, which apply to all these species and conditions except where explicitly mentioned, are of the variations that appeared (i) in the undisturbed development of certain structures: a stream, a centre, and a ring (an arrangement of cells that had the properties of a stream, though it might be found at the centre of an aggregation); and (ii) in response to a few elementary manipulations: the cutting of a stream, the recombination of lengths of stream in a number of geometrical relationships, and the removal of a centre.

OBSERVATIONS AND EXPERIMENTS

The development of a stream

A compact stream of uniform width, without tributaries, developed in one of the following ways.

1. The whole stream continued to flow into the centre, either without any change in its appearance, or with a slight decrease in width and a corresponding increase in depth. If there was no further supply of cells at its outer end, it disappeared in a few hours, leaving behind an impression in the agar as a

record of its passage. A slight variation involved the longitudinal splitting of the stream without interruption of the lines of cells.

2. The stream broke transversely at one or more places. This happened most commonly in the thinnest streams. The consequences were the same as those of experimental cutting (described below).

3. The stream developed a number of swellings (fig. 1, A, B), which were at first only slight and gently tapered thickenings. Sometimes a swelling became more and more clearly defined, growing higher, wider, and nearly hemispherical, as it involved greater numbers of cells, and slowing down and finally coming to rest as a mass identical in form, in properties and, if not too small, in further development, with the centre of an aggregation when a few hours old (fig. 1, C, D). The stream usually broke just in front of such a secondary centre. In other cases, a swelling, large or small, continued to flow towards the primary centre, maintaining a nearly constant shape and speed; or it went on developing into a secondary centre but did not quite establish its independence before reaching and fusing with the primary one; or again, it retraced its developmental path and gradually merged into the stream, in the extreme case disappearing without trace.

As many as a score of swellings might form on a stream, developing independently of one another, appearing and disappearing at different times and in any order, and as a result irregularly bunched or widely spaced and at any distance from the centre. As they moved at different speeds, one of them might catch up and fuse with the one in front of it.

When the stream developed many secondary centres, a few of the connectives between them might remain intact for some hours after the others had parted. If such a tenacious connective was cut, the cells ahead of the break would flow on into the next centre within a few minutes; presumably they had been prevented from moving by their attachment to the stationary cells in the centres behind them.

4. Spontaneous reduction of intercellular stickiness and acrasin secretion led to the more or less complete separation of the affected part of the stream into its constituent cells (fig. 1, E). The incidence of such *disintegration* was very varied: it might involve the whole stream or any section of it from its central end to its outermost part, including its swellings, though these less often than the rest. Disintegration might take more than an hour to spread outwards along the stream, stopping anywhere, or all the cells in a large section might be affected within about a minute of each other: the former was more common in *P. violaceum*, the latter in *D. discoideum*. The cells released were free to respond to any neighbouring source of acrasin.

5. While still integrated, the cells reoriented in response to a different crasin gradient. This was infrequent in compact streams and commonest in he earliest stages; it is discussed elsewhere (Shaffer, 1957 *a*, *c*).

The social scale

In some respects it was helpful to arrange formally the various states that the amoebae might pass through during the first part of their social phase in a linear series—a kind of social scale. In ascending order, these states comprised: the separate cells that were insensitive to acrasin, those that were sensitive to it, the streams, the stream swellings, and finally the centres. In general, development might be in either direction along this scale: it was upward when stream cells entered swellings or primary centres, or when stream swellings turned into secondary centres; downward when swellings shrank into their streams, or when swellings or streams separated into their constituent cells.

The effects of cutting a stream

To cut a stream, a glass rod was dragged across it and the displaced cells carried away. The effects of this operation were superimposed on the variety of developments open to uncut streams and were the same as followed spontaneous breakage. The behaviour of the section nearer the centre was almost unaltered, except when it had been slowed down by the cells behind it and increased speed after its release. The cut end of the section further from the centre behaved in one of the following ways.

1. It began to form a secondary centre at the site of the operation. Stream swellings, which had already begun to slow down and transform into secondary centres, came to rest more frequently than did the ends of uniform streams when parted from the cells that were leading them.

2. It flowed onward some distance before stopping to form a centre.

3. It flowed on fast and far enough to catch up the central stump and reunite the stream (fig. 1, F).

4. Either at the site of the cut or somewhere beyond it, it looped backwards, fused with the side of the length of stream it was leading, and thus formed a

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FIG. 1 (plate). Living cultures. The scales represent 200 μ. A, *D. discoideum*. *1*: a stream with swellings in various stages of development. One near the upper bend moves forwards short distance, then stops and transforms into a secondary centre (*2*). The two swellings following enter it; those ahead flow into the centre on the right. A third centre forms in th stream, bottom left: it is not at so advanced a stage. 1½ h between *1* and *2*.

B, *D. discoideum*. Stream swellings. Most of those visible in *1* move downwards out of th field (*2*, 1 h later).

C, *D. discoideum*. Two secondary centres, developed from stream swellings, still joined b the shrunken stream. They are no longer hemispheroidal but have central mounds ringed b shallow depressions.

D, *P. violaceum*. A line of separate secondary centres recording the position of the strean from which they derive.

E, *D. mucoroides*. *1*: a length of stream disintegrated (*d*). 15 min later (*2*), disintegratio has spread out along the tributaries of the original stream and also involved a neighbourin stream system. Most of the cells released are now more dispersed, but reintegration (i) ha already begun, and 15 min later (*3*) many of them are in streams again. Soon (*4*) nearly al of them have become reintegrated.

F, *D. purpureum*. Of three lengths cut off a stream (*1*), that nearest the centre continues t advance, following the stump of the stream (*2*), and enters the centre (*3*); the middle on disintegrates (*2*), and many of its cells disperse, though some of them follow the first lengt (*3*) and are reintegrated into a short stream (*4*); and the third one disintegrates too (*2*), bu among its cells there arises a new primary centre (*4*). *1*: 5 min after cutting. Intervals betwee exposures: 13, 15, and 25 min respectively.

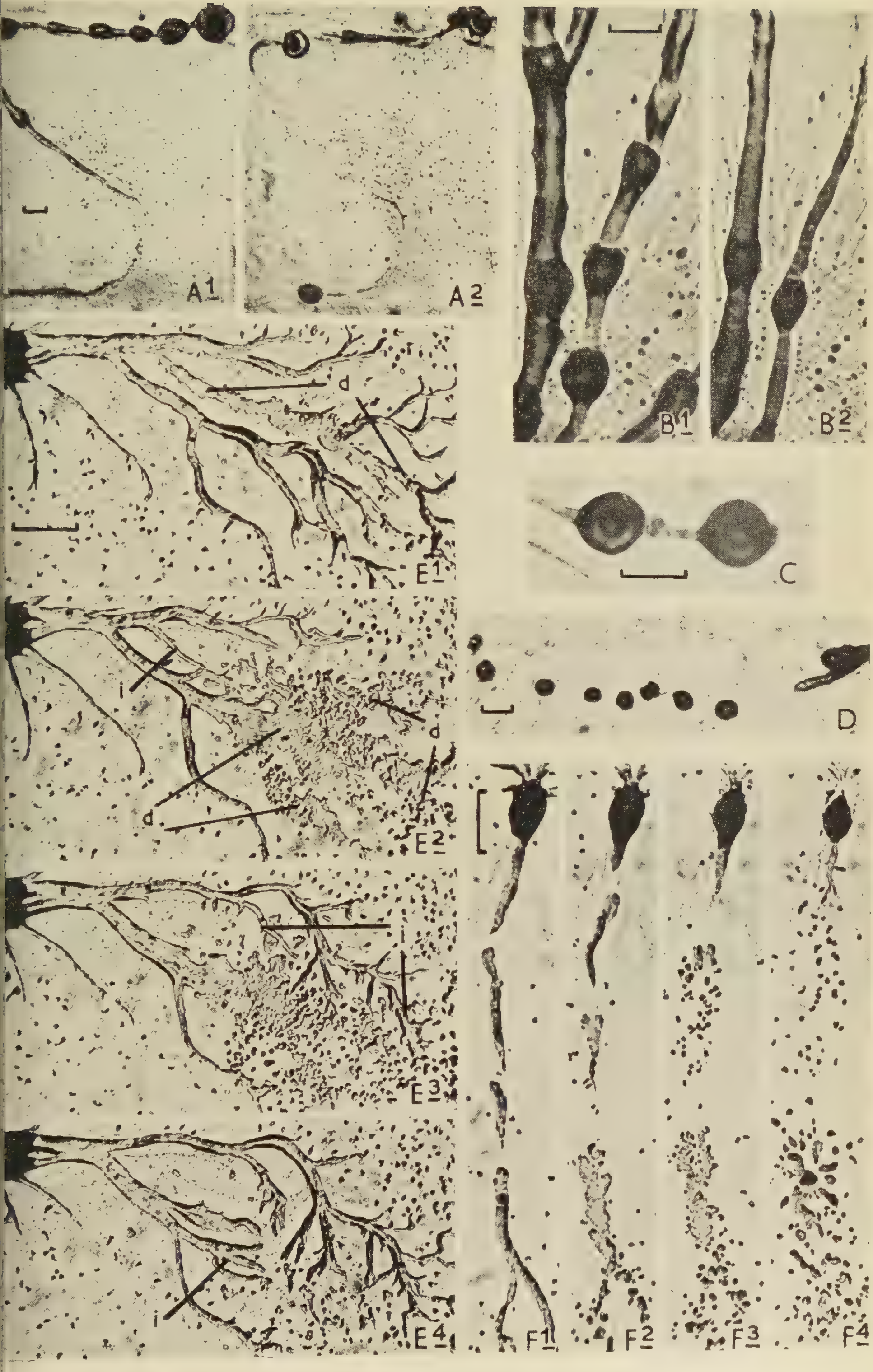

FIG. 1

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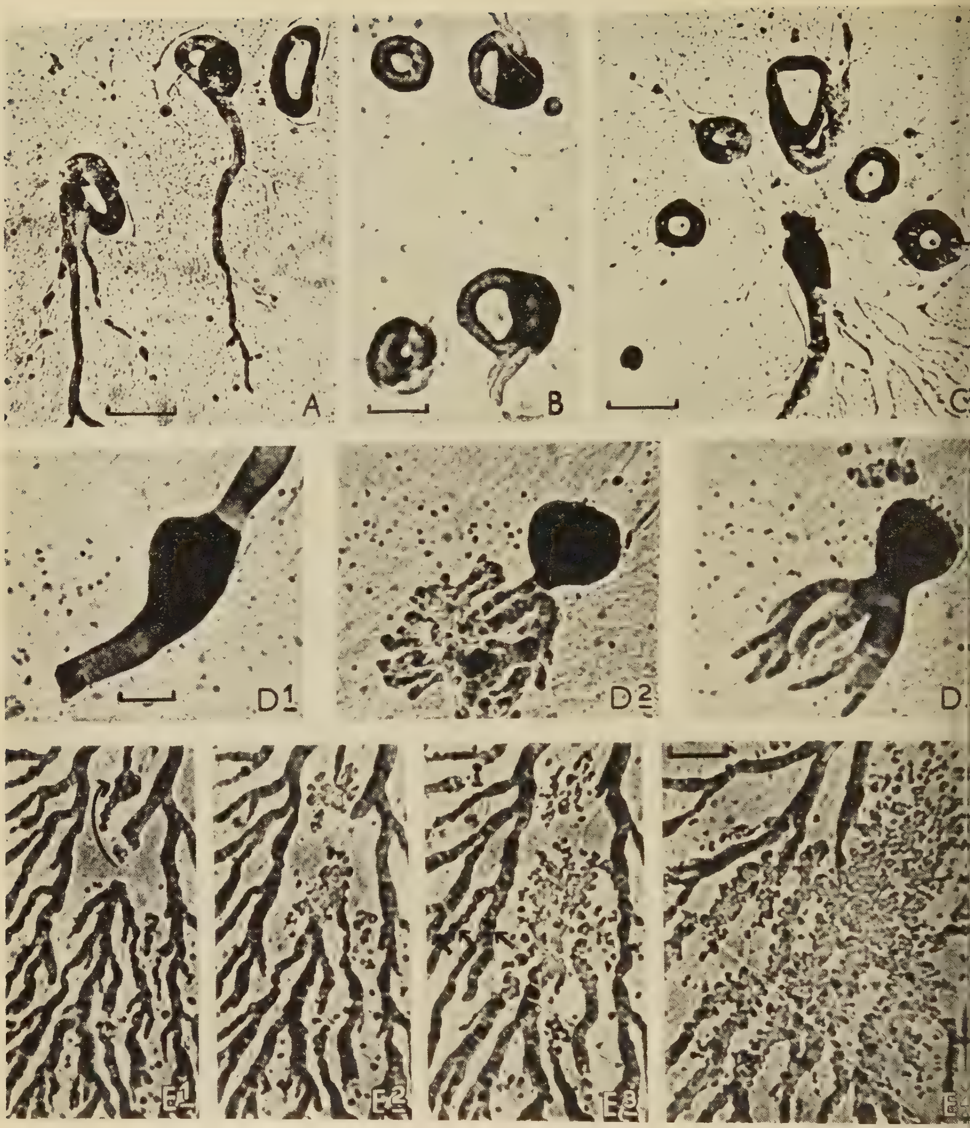

Fig. 2

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closed ring (fig. 2, A–C). This grew thicker and more solid as the stream wound itself up in it; and as the cells crawled round and round, they encroached progressively on the hole in the middle. One aggregation might develop a score of such rings after multiple spontaneous breakage of its streams.

5. Disintegration (figs. 1, F; 2, D, E) spread slowly outwards for some distance or, a few minutes or perhaps half an hour after cutting, affected simultaneously all of the section or a large part of it. It was not possible to say with certainty that there would have been no disintegration had the stream not been cut; but bearing in mind when and where it occurred, a causal relationship was overwhelmingly probable in cultures where spontaneous disintegration was rare: when it was very common, the effect of cutting might indeed be obscured. As a further complication there was some evidence, again based on probability, that the disintegration of one stream, whether spontaneous (fig. 1, E) or experimentally induced (fig. 2, E), favoured the disintegration of those next to it, and even, to some extent, of the part central to the section affected. One stream might begin to attract the cells released from its neighbour but disintegrate before they had arrived.

Of the cut ends tested, virtually all that crawled forward without an acrasin source in front of them, and also some of those that did not, were attracted to a transplanted source. As stream sections continued to secrete acrasin, both stream reunion and the formation of rings were accounted for: in these processes, depending on the configuration of the sections and their relative speeds, a sensitive cut end was guided either to the stream in front of it or to that behind it. Occasionally, however, fragments of the thinnest streams—single lines of cells—flowed onward but were unresponsive to transplanted sources. These few cells had probably wandered into an aggregation and were perhaps completely insensitive or of such low sensitivity that they would only respond to a source if they made actual contact with it.

The leading ends of stream sections could be classified retrospectively on

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FIG. 2 (plate). Living cultures. The scales represent 200 μ. A, *P. violaceum.* Rings formed at the central ends of stream lengths that broke off spontaneously. The ring on the right has already absorbed all of its source stream; that in the middle has only a minute aperture, and the stream makes an extra half turn around it.

B, *P. violaceum.* Similar to A. Note the local swellings on the two rings on the right. The original diameter of the ring, bottom left, is marked by a circle around it in the agar.

C, *P. violaceum.* Rings formed spontaneously on five streams that all led to the centre in the middle.

D, *D. discoideum.* *1*: A stream, flowing downwards, cut just ahead of a swelling. *2*: 1 h later. The part of the stream following the swelling has entered it, and the short section ahead of it has disintegrated and begun to reorient to it. *3*: 30 min later. The cells released have been reintegrated into streams flowing in the opposite direction.

E, *P. violaceum.* *1*: the middle stream has been cut, the cells removed being pushed towards the top of the field. *2*: 15 min later. Disintegration has affected the heap of displaced cells and is beginning to spread upstream from the cut. *3*: 15 min later. More of the same stream system has disintegrated. *4*: 35 min later. The field has been shifted downwards about $\frac{1}{4}$ of its height. Disintegration has spread further along the cut stream and also occurred spontaneously in the streams on the left of it. Later still (not shown), the cells released enter the two intact streams bordering the area of disintegration.

a scale according to whether they entered, or approached, a higher or a lower social state. At the top could be placed those that did not move even towards an external source of acrasin: they developed into secondary centres. Below them came those that did respond to a source—in descending order: those that would not move without one, those that would, and those that disintegrated. At the bottom there were the few that moved but were not sensitive.

Though it was not possible, after visual inspection, to predict with certainty just what would be the behaviour of a given stream, whether entire or cut, yet one could say that the larger the ratio of its depth to its width, a measure of the packing and bonding of its cells, the greater the probability, in any one species, that development would lead up the social scale. *If* the ratio varied considerably along a stream, it tended to be highest where the cells had been integrated for the longest period.

The recombination of lengths of stream

If moderately thick streams flowing over an agar surface were covered with a drop of water, they could be cut up into suitable lengths, which could be lifted and recombined in various ways before the water was absorbed. The effect on a stream of submersion, drying, and the associated mechanical shock, without any further experimental interference, was to encourage disintegration, breakage, and the formation of secondary centres; but there were cases when even a whole stream continued to flow forward uniformly, and parts of one often did so.

If an isolated uniform stream was cut into sections and these were recombined in any order, though with the same polarity, all the cells in it might continue to flow forwards towards the new front end, and this behaved like that leading any ordinary cut stream.

If the section in the rear was very short and was reversed, it sometimes disintegrated and allowed its cells to turn and move individually towards the next section; or it contracted into a heap, which developed into a secondary centre or gradually entered the rest of the stream, and which in either case tended to act as an anchor restraining the movement of the cells in front of it.

If the reversed section in the rear was considerably longer, it flowed away from the section to which it had been joined, though if an appreciable number of cells had been overlaid by others polarized in the opposite direction, the movement of both groups was hindered. In this case, a swelling formed at the junction and developed into a secondary centre in that position or after some delay moved either forwards or backwards; and the stream parted on one or on both sides of it, which allowed the sections to flow according to the original polarity of their cells.

If the reversed section was not at the rear of the reconstituted stream but at the front or in the middle, the cells at a junction that united divergent polarities behaved as just described; and those at a junction that united convergent polarities formed a centre there or flowed out sideways for some distance.

The over-all patterns that developed when a length of stream was laid at right angles across an intact stream were of great variety; of these, some are represented in fig. 3, excluding those in which disturbances of stream uniformity could not reasonably be attributed to the special features of the experiment. The stream cells tended to crawl onwards without changing their polarity; at the crossover, those from the rear arms could continue in their original direction, or be guided by the flow at right angles, or stop at this ambiguous signpost and there form a secondary centre. The leading end of the cross-stream, when it went on moving, often looped back towards one of the three nearest arms of the cross and on reaching it was directed by the sense of flow therein (fig. 4, A). The tangle was sometimes resolved by disintegration (fig. 4, B); at other times, one or both of the streams parted, frequently just ahead of the crossing (fig. 4, A). If the leading arm of the cross-stream was very short, it commonly contracted into a clump next to the main stream and sometimes flowed 'backwards' into it; it did this, if at all, only after a delay, being long preceded by the other arm, which needed no repolarization.

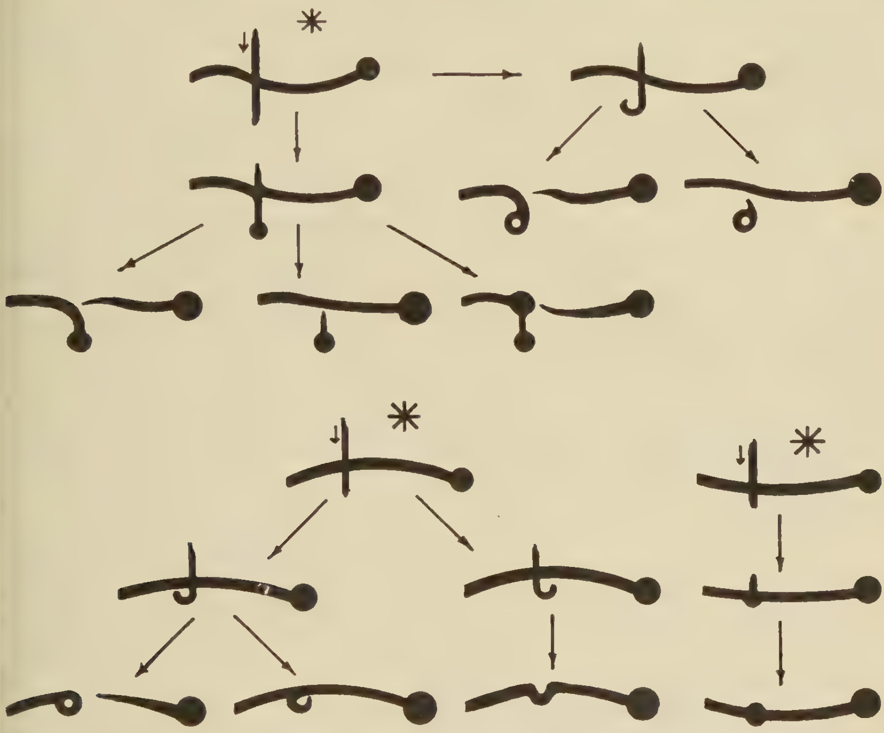

FIG. 3. Diagram of some of the results obtained when a cut length of stream (represented as flowing downwards initially) was laid across an intact stream

It appeared that all these manifestations of stream polarity could be adequately accounted for in terms of the orientation of the polarized stream cells, which tended to be self-preservative.

The effects of removing the centre

Excising the centre of an aggregation did not have any consequences beyond those to be expected from cutting all the streams (though the possibility of there being an effect on rhythmic movement was not examined), except in the earliest aggregations, where further association of the cells might be frustrated temporarily or, at least in *P. violaceum*, even permanently. Each cut stream followed any of the developments already listed; and in addition all the products interacted as determined by their spatial configuration, motility, acrasin secretion and sensitivity, and adhesiveness. The resultant patterns were of endless variety.

The development of the centre

An entire centre, whether primary or secondary, might spontaneously revert to the unintegrated state (fig. 4, C), and amoebae released from it had the same properties as those from disintegrated streams: they were sensitive to acrasin and did not secrete it, and their degree of separation depended on their residual stickiness, and this in turn on the species and the environment (Shaffer, 1957*a*). On occasion, very many of the multitude of minute centres that had appeared in a culture of *D. purpureum* or *P. violaceum* disintegrated, even when there were no other centres near them which might have been competing with them or to which their cells could be attracted on their release. These cells, if they did not re-enter an aggregation (either one formed near by or one started by themselves), sometimes soon lost their sensitivity to acrasin.

A less extreme change of state was for a centre to assume the properties of a stream: it began, sometimes quite suddenly, to flow directly towards another acrasin source, often one that had been its close and unstimulating neighbour for some hours.

FIG. 4 (plate). Living cultures. The scales represent 200 μ. A, *D. discoideum.* A cut length of stream, flowing upwards, was laid across an intact stream, flowing to the right. *1*: the front end of the cross-stream, having made a U-turn to the right, is about to enter the main stream. Much of the rear arm of the cross-stream has turned right at the cross-over and thickened the main stream. *2*: the main stream has parted just to the right of the cross-over, and the section to the left of it now advances through the U-shaped diversion laid down by the cross-stream.

B, *D. mucoroides.* A short length of stream, flowing upwards, laid across an intact stream, flowing to the right. The leading arm of the cross-stream disintegrates (*2*), and its cells form streams flowing back into the main stream (*3*). Meanwhile, the rear arm of the cross-stream, whose cells had no need to reverse their direction of movement, has already entered the main stream.

C, *D. purpureum.* A field of centres, mostly without streams. Three (*a*) are solid masses; one (*b*) is disintegrating and still contains a clump of well-packed cells; and one (*c*) is absorbing its neighbour, which has changed into a stream and in turn is collecting cells from a disintegrated centre (*d*). The sites of three other disintegrated centres (*e*) are clearly visible. Two of the masses (*f*) are now rings with minute apertures.

D, *P. violaceum.* A ring on the point of closing at the front of a length of stream.

E, *P. violaceum.* An irregular and complex ring formed by a stream.

F, *P. violaceum.* A figure of eight formed by streams.

G, *D. mucoroides.* A ring fabricated from a stream.

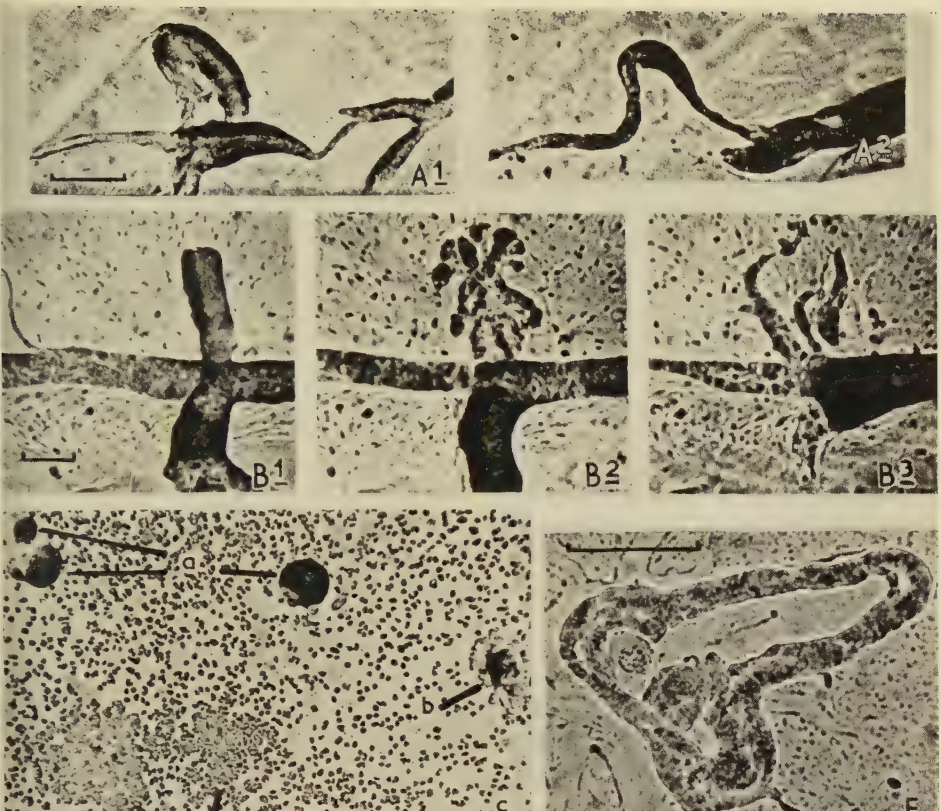

Fig. 4

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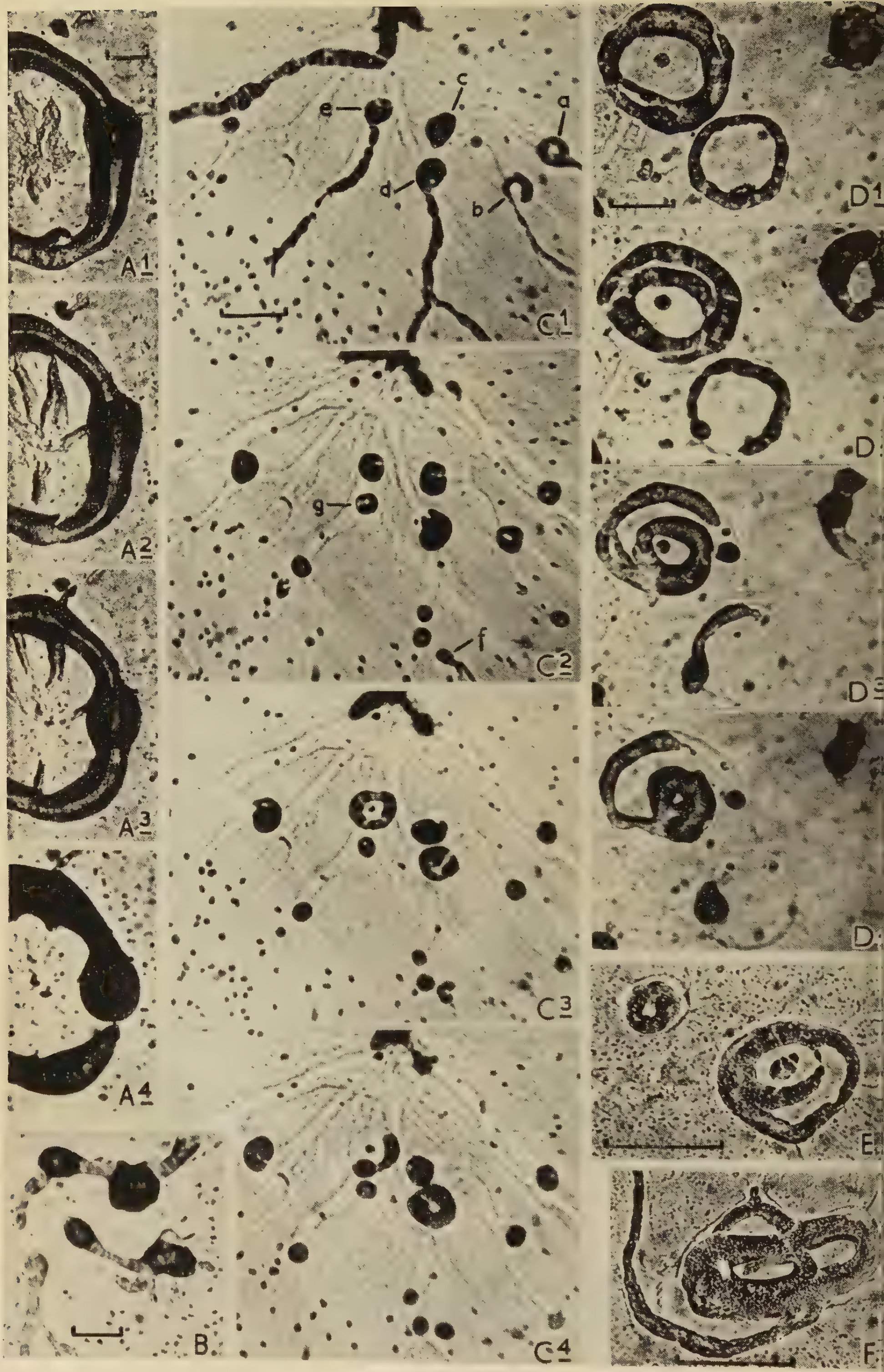

FIG. 5

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The development of a ring

Before describing how circularly polarized organizations continued their development, an account will be given of their formation and properties.

Irregular loops and rings appeared in the preplasmodium of *D. mucoroides* (a stage just preceding true aggregation), but they were short-lived, because the level of intercellular stickiness was too low to co-ordinate the movements of the cells to any great extent or to prevent their escaping partially or completely from a number that chanced to come in contact. To produce a more lasting ring, there had to be factors for uniformly aligning the polarized components, preserving this alignment, closing the ring, and keeping it closed.

In the type of ring-making that was described earlier in this paper, the first step, which produced and preserved the required alignment, was the formation of a stream. If this structure broke in two spontaneously or was cut, the exposed end further from the centre, if sensitive to acrasin, could be drawn back to one side (fig. 4, D) till it finally made contact with the cells it was leading, if it was stimulated sufficiently asymmetrically by the acrasin they produced. If the stream was initially symmetrical, this stimulation could occur only if the end first deviated enough by chance. The narrower the stream, the fewer the cells, bound side by side, in the lead; so the greater the probable extent of their deviation and the higher the proportion of their sensitive surface exposed to the external gradient. Of the motile isolated lengths of stream studied, those that were single lines of cells almost always formed rings, whereas the wide ones not infrequently crawled straight ahead.

Sometimes the end made an extra half turn before closing the ring, thus

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FIG. 5 (plate). Living cultures. The scales represent 200 μ. A, *D. mucoroides*. Response of acrasin-sensitive cells placed in the middle of a fabricated ring. (These cells initially cover too large a fraction of the central space in a single heap for their orientation to be considered to be more than a rough indicator of the acrasin gradient.) The ring opens directly ahead of the secondary centre it develops (*4*).

B, *D. purpureum*. A ring with four swellings. Only two of the connecting arcs remain. An inflowing stream has parted at one point.

C, *D. mucoroides*. Rings developing spontaneously in one aggregation, whose centre (from which a fruiting body rises into the air) is at the top. The central space in ring *a* disappears (*3*) and reappears on the other side after rotation of the mass. Ring *b*, just closing (*1*), condenses into a solid mass. Condensed ring *c* re-forms a central space (*2*); the new ring opens at one point (*3*) and after absorbing the ‘tail’ again becomes a solid heap. Ring *d* enlarges its hole and opens; its two ‘tails’ (*2*) are the ends of the two concentric arcs into which this part of it has cleaved; a new hole appears in the mass (*3*) and revolves with it (*4*). Ring *e* re-expands considerably (*3*), opens at one point, and flows into a secondary centre. Condensed ring *f* (*2*) expands and simultaneously opens (*3*). The small hole in ring *g* (*2*) rotates with it. A number of other rings are visible. Intervals between exposures: 68, 40, and 12 min respectively.

D, *P. violaceum*. Rings formed spontaneously. Bottom: the ring develops a single swelling and opens just ahead of it (*2*). Right: the condensed ring re-forms a central space (*2*) and opens (*3*); one of its swellings fuses with the one in front of it (*4*). Top left: parts of the ring split into concentric arcs; the outer ring opens at one point (*2*) and again (*3*) ahead of a secondary centre; the inner ring contracts (*4*).

E, F, *P. violaceum*. Complex rings formed by cleavage.

describing a short spiral (fig. 2, A); sometimes the ring was irregular in shape (fig. 4, E). Rarely, two rings formed so close together that small shifts in the paths taken by their cells sufficed to bring them into contact. If they were rotating in opposite directions, a figure of eight resulted, after their streams had entered them (fig. 4, F).

Contrasted behaviour was shown by a short chain of preaggregation cells or de-integrated ones: its leading cell might turn back towards those following it and then turn away again, cross over them, or crawl alongside them in either direction.

The radius of a ring formed on a stream was commonly little more than twice and not often more than four times the width of the stream. A very much larger one (fig. 4, G) could be produced by floating a number of lengths of stream off the agar and joining them up with their polarity uniformly clockwise or anticlockwise. A fabricated ring developed like a self-made one, and its large size facilitated the study of its properties. If the polarity had been reversed in any section, the ring parted at the junction where the cells pointed away from one another.

The origin of some rings that did not develop from established streams was to be sought in the earliest stages of aggregation. At times, a field of cells that were not in contact with one another was oriented by a chain reaction passing from cell to cell (Shaffer, 1957*b*). If this did not spread uniformly through the field, the pattern produced, which was an approximate record of its progress, might in a limited area be circular instead of radiate (Shaffer, 1957*c*). When this was so, the cells there might eventually condense into a compact ring, which developed much like one formed from a pre-existing stream. It was fed by streams that entered it tangentially, but their curvature, if any, was unrelated to the direction in which it rotated, having been determined by local conditions as the reaction spread further.

Cells in a compact ring, like stream cells, could in general respond to a natural acrasin source external to it only if their orientation was disturbed or if their front ends were exposed by cutting or spontaneous breakage or by disintegration.

If acrasin-sensitive cells in heaps were arranged so as to encircle a ring or were placed on the bare agar in the middle of a large one (fig. 5, A), they commonly moved approximately radially towards it, inwards and outwards respectively.

A ring proved to be comparable with a straight stream not only in its properties but also in the types of development open to it. At one behavioural extreme, swellings appeared on it and immediately transformed into secondary centres. The ring usually broke just ahead of each of them, though often at widely different times (fig. 5, B). If there was only one centre, the cells behind it flowed into it directly, whereas those immediately in front had to crawl right round the ring to reach it (fig. 5, A*4*). When the tendency to transform into a higher social state was less marked, the swellings did not come to rest and form centres for some time; or no swellings appeared at all, and instead the ring remained of uniform thickness and perhaps broke at some point, as a stream might do, or slowly decreased in diameter as it revolved till there was no central hole left. In the latter case, the solid mass produced then usually behaved like an ordinary centre. But sometimes, and most commonly when it was very small, a centre made by the condensation of a ring re-formed a hole in its middle, which gradually enlarged as the mass rotated (fig. 5, C, D). This reversal of development apparently corresponded to the conversion into a linear stream of a centre that had been solid from the beginning. Only rarely in this respect did one type of centre develop like the other. Further behaviour that led to a lower social state was the shrinkage of a swelling until it was indistinguishable from the rest of the ring, and partial or total disintegration at any stage.

At times, some portion of a ring cleaved into two or more concentric arcs (figs. 4, E; 5, E, F)—the counterpart of the longitudinal splitting of a straight stream. Each arc might develop differently (fig. 5, D) and quite complex patterns be produced.

Variations in the environment

The full range of development described was observed in cultures kept at temperatures of 10°–28°, on agar of pH 4–8 (0·01 M phosphate buffer or McIlvaine's buffer as used by Hirschberg and Rusch (1950)), and in bright light or total darkness (as far as could be checked by examining culture plates immediately they were exposed to light). The most marked effects were produced by changes in humidity: they can be summed up by saying that in general the drier the environment, the greater was the proportion of cells that were at any moment approaching a higher social state, and the more rapidly did they do so. Return to a lower state, as for example disintegration following stream cutting, was most common under water. Nevertheless there was a great variety of behaviour over a wide range of humidities, and even the permanent removal of the culture-plate lid during aggregation might be followed by considerable disintegration of the flatter streams.

DISCUSSION

If the literature on the aggregation of the four species here studied is taken as a whole, it reveals that with or without experimental interference there may be considerable variation in behaviour. Arndt (1937) reported that in *D. mucoroides*, especially in the early stages of aggregation, a succession of centres might gain control over a field of amoebae, and that some centres were absorbed by the others. Raper (1940*a*) found that the amoebae of *D. purpureum* might enter and re-enter different spheres of organization before being incorporated in the well-defined streams of a determinate aggregation. But once amoebae were in such a radiate aggregation, they typically proceeded without interruption to its centre, though breaks did appear and disappear again in the smaller streams (Raper, 1941). A break in a stream of a later stage usually persisted, the cells in the section further from the centre piling up at its broken end as they would have done at the centre. This behaviour could be induced in *D. discoideum* by slightly drying a culture or raising its temperature (Raper, 1940*b*), but it commonly occurred in *P. violaceum* even without this treatment (Raper, 1940*a*). In my experience, changes of state in general, down as well as up the social scale, are more frequent in *P. violaceum* than in *D. discoideum*. Arndt (1937) described local dilations of a stream moving along it; these were apparently what in this paper have been called stream swellings; one of them 'burst' into fragments that were attracted to another centre. Arndt (1937), Raper (1941), and Bonner (1950) saw aggregations in which the cells formed a hollow revolving ring at the centre instead of a solid mass. Arndt and Bonner reported that as the ring rotated, the whole it enclosed decreased in diameter till finally it disappeared: and Raper, that the amoebae concentrated at one point on the ring, on which the streams then converged.

Arndt, using *D. mucoroides* cultured on agar, cut a stream by laying a glass needle across it; he found that if the leading end of the section further from the centre managed to crawl past this barrier, it was unable to catch up the rest of the stream; it soon stopped moving and the cells behind it piled up on it. Raper (1940*b*) found that the outer section of a cut stream of *D. discoideum* flowing on agar collected at the point where the break was made. Bonner (1950) noted that separated lengths of a stream of *D. discoideum* under water broke down into their constituent amoebae. Raper (1940*b*) removed the centre of a *discoideum* aggregation on agar and found that the streams piled up at their cut central ends and eventually formed fruiting bodies; a film made by Bonner (1944) shows this operation under water being followed by dispersal of the whole aggregation.

Bonner (1950) encountered variation when he removed the centre to which some amoebae were crawling under water and placed it directly behind them: each of them either first rounded up and then sent out a new pseudopodium towards it or made a U-turn while still elongated. If he cut a short length off a stream, reversed it, and stuck it on to the central stump, it shortened as its cells rounded up and was later drawn 'backwards' into the centre.

These descriptions and the material presented in this paper show that slime moulds can behave in a variety of ways that at first sight is rather bewildering. In trying to make a pattern of them, it is helpful to recognize that a cell in this developmental stage can exist in one of four main states (Shaffer, 1957*a*): (1) when it is but slightly or not at all adhesive to similar cells, does not secrete acrasin, and is unable to orient in an acrasin gradient; (2) when it can orient but is otherwise effectively as in (1); (3) when it is strongly adhesive, acrasin-secreting, elongated, and guided by an external acrasin gradient if there is one and by the orientation and direction of flow of its neighbours if there is not (the stream cell); (4) when it is strongly adhesive, secreting, effectively non-motile, and nearly isodiametric (the centre cell). The state of a cell in a stream swelling is intermediate between (3) and (4).

Apart from the quantitative variation of certain properties, such as velocity and acrasin secretion, within a state, the entire repertory of the cells is essentially limited to their entering, or approaching, these states in various sequences and at various rates. The transformations that have been described in this paper and the last are from the first state to the second; from the second to the first, third, and the fourth (which includes primary-centre formation); from the third to the second, and to the fourth (which includes the formation of secondary centres); and from the fourth to the second and third. Most of these transformations may occur over a wide range of environmental conditions, though drying strengthens the drift to a higher state.

It is suggested that variability is occasioned by the simplicity of the mechanisms controlling development and the absence of some that are essential if more complex morphogenesis is to be achieved.

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Histochemical Studies of Lipids in Oocytes

I. Lipids in the Oogenesis of *Columba livia*

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Summary

Judging from their sizes and histochemical reactions, three types of lipid bodies in the form of granules and spheres have been observed in the early stages of oogenesis. The minute granules (L_1), belonging to the first type appear to consist of phospholipids, triglycerides, and fatty acids, but the presence of proteins cannot be ruled out. They correspond to the so-called granular mitochondria of other authors of papers on the oogenesis of birds. The second type of lipids (L_2) are of medium size and they contain phospholipids and a certain amount of triglycerides. They resemble the so-called 'Golgi bodies' of earlier authors in appearance and react like them with osmium tetroxide and silver nitrate. They seem to originate from the granules of the first category (L_1). The third type of lipids (L_3) are the AH-negative spheres consisting of triglycerides and cholesterol and its esters. They correspond to the 'fatty yolk' of earlier authors. Their origin appears to be from the second type of lipid bodies (L_2), as intermediate bodies (*int*) of varying chemical composition between the L_2 and L_3 have been observed. The 'peripheral lipid bodies' (*pl*) found in association with vacuoles are larger than the second type of AH-positive bodies (L_2), but they seem to contain different types of phospholipids, triglycerides, and some other substances also. Besides these lipid bodies, a mass of lipoprotein bodies has also been observed. At the time of their origin, the yolk globules of the advanced oocytes do not contain lipids but later on they develop triglycerides and phospholipids. The lipid bodies of the follicular cells correspond very closely to the first two categories of sudanophil AH-positive bodies (L_1 and L_2) of the oocyte proper.

Introduction

SINCE very little work has been carried out on the histochemistry of lipids in oocytes of animals by modern histochemical techniques, it is intended to work out the histochemistry of these lipids by techniques whose validity is now well established. This paper on the lipids of the pigeon oocytes is the first of the series.

To the best of my knowledge there are only three previous publications on the subject. Marza and Marza (1936) and Singh (1938) have demonstrated histochemically fats, lipids, and cholesterols in the hen's egg, and the presence of cholesterol bodies in the pigeon oocytes respectively. But most of the techniques employed by the former authors are not considered valid by modern histochemists such as Cain (1950) and Pearse (1954). Nama (1956) finds that, in the oocytes of *Columba livia*, the 'Golgi bodies' contain phospholipids, lipoproteins, and proteins, associated with triglycerides and cholesterols. It appears that this author has confined himself to the cytochemistry of Golgi elements of a particular stage only in oogenesis. Apart from this, Nama

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does not seem to be clear with regard to the identity of his Golgi elements and phospholipids scattered in the cytoplasm.

In the present paper three distinctly lipid bodies of different chemical nature have been described in the early oocytes of the pigeon. For the sake of convenience they have been distinguished as L_1, L_2, and L_3. The changes undergone by these bodies in the course of oogenesis have also been followed very closely. The lipid contents of the yolk of advanced oocytes have also been studied. The histochemistry and behaviour of a fourth type of lipid bodies (*pl*) found at the periphery of the oocyte in close association with some non-lipid vacuoles have also been studied. The lipid contents of the follicular epithelium have been analysed.

An attempt has been made to correlate the cytoplasmic components after chrome-osmium and silver nitrate techniques with the usual histochemical techniques. Most of the chrome-osmium and silver techniques have been found to preserve only a very small portion of the total lipid contents of the cell, which, in this particular case, are dominated by neutral fats (triglycerides).

MATERIAL AND METHODS

The specimens of the pigeon, *C. livia intermedia*, were collected from Hoshiarpur proper. Only adult specimens were used. The animals were dissected immediately after the head had been chopped off. The ovary was removed in physiological saline (Baker, 1944). After the blood had been washed off, the ovary was cut into small pieces and placed in various fixatives.

The techniques for morphology

The following techniques were employed for studying the morphology of cytoplasmic components. The material was fixed according to Lewitsky (Flemming without acetic acid), Champy, Bouin, Aoyama, Da Fano, Mann-Kopsch, and Kolatchev techniques and embedded in wax. The sections were either stained with iron haematoxylin or mounted unstained as in silver nitrate and osmium tetroxide techniques. The silver nitrate preparations were also toned with gold chloride to remove the excess of silver.

Histochemical colour tests

For all the histochemical techniques, gelatine-embedded material fixed according to the methods given below was employed.

Sudan black B in 70% ethanol was used for the demonstration of lipids in the material fixed in formaldehyde-saline (Baker, 1949) and formaldehyde-calcium (Baker, 1946) with or without post-chroming. Neutral lipids (triglycerides) were demonstrated by the Nile blue technique of Cain (1947 *a*, *b*, 1948). Phospholipids were demonstrated by the acid haematein technique with pyridine extraction control (Baker, 1946, 1947; Cain, 1947 *a*, *b*, 1950; Casselman, 1952; Pearse, 1954). Only those lipids that were positive in acid-haematein

(AH) and negative in pyridine extraction (PE) will be described as AH-positive.

The Sudan III and Sudan IV method of Kay and Whitehead (1941), the gelatine-Sudan method (Govan, 1944), and Fettrot (of Ciba) in 70% ethanol as described by Pearse (1954), were employed for neutral fats (triglycerides). Only those bodies which gave a pink colour with Nile blue and were stained with these dyes, have been interpreted as containing neutral fats. I used Sudan III and Sudan IV in the gelatine-Sudan method of Govan. The gelatine-Sudan method and Fettrot gave a very clear picture of the lipids as there was no precipitation of the dyes; nor was there any loss of small lipid droplets in the former technique.

Fresh coverslip preparations of the contents of the ripe ovum treated for a short period with 2% osmium tetroxide solution as used by Nath since 1929 (see Nath, 1957*a*, for full references) were also used for the study of lipids. Fischler's method for fatty acids as reported by Pearse (1954) with usual control was also used. Schultz's method for cholesterol and its esters as reported by Gomori (1952) was used on gelatine sections oxidized by iron alum.

Solubility tests

Acetone was used to dissolve neutral fats and cholesterols, but the phospholipids were removed with alcohol and ether. Boiling alcohol was used to extract the lipoproteins.

For solubility tests, sections of material fixed in formaldehyde-calcium was used, with or without post-chroming.

Pieces of fresh ovary were also extracted for 24 h with acetone to dissolve triglycerides and cholesterols, or with alcohol and ether to dissolve phospholipids. Some of the extracted material in each case was fixed in formaldehyde-calcium, post-chromed, and embedded in gelatine for the cutting of sections, while the rest of the material after extraction was taken directly to water by passing it quickly through various grades of alcohol. From water it was embedded in gelatine for sectioning. After extraction of the fresh material, fixation in formaldehyde-calcium with post-chroming did not make any difference in the preservation of lipids in the material under investigation. The lipids that resisted cold acetone also resisted hot acetone.

For the extraction of lipoproteins (which resisted cold acetone, alcohol, and ether) boiling alcohol was used for 16 h and gelatine sections were prepared.

The cold and hot ether did not give good results in the case of fresh material. This may be attributed to its inability to penetrate the tissue. For the solubility of various lipids I have not noticed any difference between material fixed in formaldehyde-calcium and fresh tissue, but the great contraction of the oocytes and the displacement of various lipid bodies are disadvantages in using fresh tissue. In the case of material fixed in formaldehyde-calcium and post-chromed, the phosphlipids resist the action of fat solvents named above.

OBSERVATIONS

The sudanophil lipids in the early oocytes of the pigeon are present in the form of granules and spheres which can be divided roughly into three categories according to their size and histochemical reactions.

1. *The minute granules* (L_1). These are sudanophil and AH-positive, give a light pinkish tinge with Nile blue, and appear orange-red with Sudan III and Sudan IV, pinkish-red with Fettrot, and deep blue after Fischler's method for fatty acids. After treatment with acetone these granules appear to lose some lipids as they colour comparatively less intensely as compared with untreated sections coloured with Sudan black B. They are still AH-positive but do not give a pinkish tinge with Nile blue. With Sudan III, Sudan IV, and Fettrot the colour that they take up is so pale that the reaction may be considered as negative. Some of these granules seem to lose their lipids completely in acetone. In alcohol and ether all these granules lose their lipid contents as they do not stain after Sudan black B (see L_1 in figs. 1, A–G; 2, A–D; and 3, A–F).

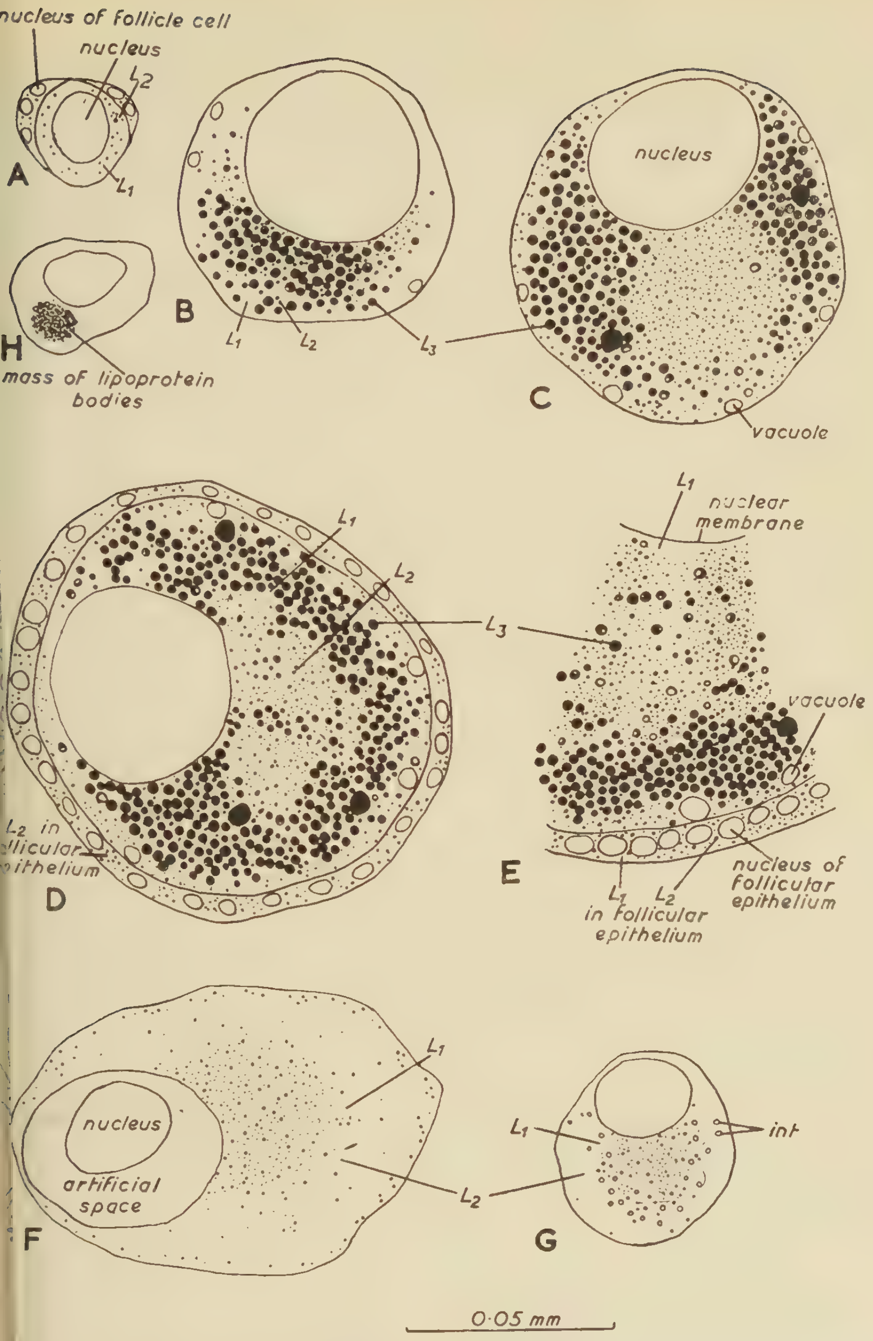
2. *The second type of granules* (L_2). These are bigger than the first ones, intensely sudanophil, AH-positive, are coloured pink with Nile blue, and appear deep orange-red after Sudan III and Sudan IV, and pinkish-red with Fettrot (see L_2 in figs. 1, B–G; 2, A–C; and 3, A–E). They dissolve completely in cold and hot acetone, alcohol, and ether, as they do not appear at all with Sudan black B after such treatments.

However, a mass of sudanophil material has been observed after treatment with these fat solvents at a particular stage of oogenesis (*lip*, fig. 1, H). This mass is only colourable with Sudan black B but not with other Sudans and Fettrot. It is also not stained with acid-haematein. When the material is boiled with alcohol, its sudanophilia is lost completely as it cannot be seen even after colouring with Sudan black B. This mass of sudanophil material will be referred to again in the discussion.

3. *The third type of lipids* (L_3). These generally occur as spheres of comparatively large size, are deeply sudanophil and AH-negative, colour red with Nile blue, Sudan III, and Sudan IV, and pinkish-red with Fettrot. These bodies are soluble in acetone, alcohol, and ether, whether the extractions are tried on fresh or fixed and post-chromed materials (see L_3, figs. 1, B–E; and 2, A–C).

Many varieties of the lipid bodies intermediate between these three forms have also been observed, which will be described in the developmental history of the oocyte.

In the earliest oocyte examined, the sudanophilia occurs in the form of a few separate granules of the first category (L_1). These granules are scattered in the cytoplasm around the nucleus, which occupies the centre of the oocyte (fig. 1, A). Development of the oocyte from now onwards reveals a considerable increase in the size of the nucleus as well as the cytoplasm, although during the later period of growth the cytoplasm outstrips the nucleus in its



G. 1. A–D, sections of oocytes prepared by the Sudan black B technique of Baker (1949). portion of section of an oocyte prepared as A to D. F, section of an oocyte fixed in Lewitsky stained with iron haematoxylin. G, a portion of an unstained oocyte prepared by the latchev technique. H, a section of an oocyte showing the mass of lipoprotein bodies left er treatment of the fresh material with cold alcohol for 24 h. L_1, L_2, L_3, lipid globules of the 1st, 2nd, and 3rd categories; *int*, intermediate bodies growth. With the growth of the oocyte the nucleus becomes eccentric and th amount of sudanophilia increases gradually. Fig. 1, B represents an oocyte i which there is present a considerable amount of sudanophil bodies. Th largest of these, which more or less dominate the picture, belong to the lipi of the third category (L_3). The granules that are somewhat smaller than thes can be assigned to the second category (L_2), whereas the minutest granule are of the first category (L_1).

It appears that the large AH-negative spheres (L_3) originate by the growt and transformation of the smaller ones (L_2). Such a conclusion is strengthene by the fact that many granules which are intermediate in size between th AH-positive (L_2) and AH-negative (L_3) sudanophil bodies, give only a sligh AH-positive reaction. Usually with acid-haematein these intermediat granules (*int*) appear in the form of duplex structures the rim of which give a slight AH-positive reaction while the medulla is completely AH-negativ (see *int*, fig. 3, A, B). With Sudan black B these bodies appear uniforml blue-black. These duplex vesicles have also been observed in Kolatchev an Aoyama preparations (*int*, fig. 1, G), although in the latter case they ar greatly contracted. When gelatine sections of material fixed in calcium formaldehyde and post-chromed are treated with cold acetone, alcohol, an ether, and coloured with Sudan black B, these bodies continue to appear i the form of duplex vesicles. These are not preserved when living material extracted with these solvents. This observation leads to the conclusion that th intermediate bodies (*int*) are composed of a cortex rich in phospholipids an a medulla rich in triglycerides.

The increase in the number of all the three different categories of sudan phil bodies keeps pace with the growth of the oocyte.

The next stage (fig. 1, C) initiates the peripheral disposition of the larg AH-negative spheres (L_3). To begin with these large spheres become arrange in two groups occupying each side of the nucleus, which has come to lie on o side of the oocyte. In between these groups of large AH-negative spheres li a concentrated mass of AH-positive sudanophil bodies belonging to both th first (L_1) and the second (L_2) categories. The intermediate forms between th second (L_2) and third (L_3) categories of lipids occur mostly around the con centrated mass of AH-positive sudanophil bodies. The large spheres (L_3) d not react positively to the AH-test (fig. 3, A, B).

The large AH-negative sudanophil spheres (L_3) now move still furth towards the periphery of the oocyte and become arranged in the form of ring immediately below the follicular epithelium (fig. 1, D, E). The number these bodies is increased greatly at this stage. It has also been observed th during the later stages of growth some of these large spheres do not colo homogeneously with Sudan black B, thus appearing in the form of rings wi varying thickness of their cortices. This appearance may be attributed eith to incomplete fixation of the more centrally situated lipids, or to the presen of lipids in the centre solid at ordinary temperatures, which remain u coloured with Sudan black B (figs. 1, C–E; 2, A). This ring-like appearance rge spheres (L_3) is of very rare occurrence and has only been observed in aterial fixed in formaldehyde-saline and coloured with Sudan black B (after aker, 1949). A similar appearance of fat droplets has also been recorded by radbury (1956).

Simultaneously with the peripheral disposition of the lipids of the third tegory (L_3), the AH-positive granules (L_1 and L_2) become dispersed in the toplasm. The granules now appear in more or less irregular patches, the termediate spaces between these patches containing widely distributed ranules (fig. 1, E).

The large AH-negative sudanophil spheres (L_3) are not preserved in ny of the chrome-osmium or silver nitrate techniques. But when unstained ewitsky preparations are examined immediately after mounting in Canada alsam, most of these AH-negative spheres are seen as black globules. hen such slides have been dried in the oven, these black globules are completely washed out and appear in the form of vacuoles.

The smaller AH-positive sudanophil granules (L_1 and L_2), on the other and, are preserved in chrome-osmium and silver nitrate techniques (fig. 1, F,), and in unstained Lewitsky and Champy preparations they are seen as dark ranules.

In the chrome-osmium and silver nitrate techniques the nucleus and the toplasm of the oocytes undergo great contraction and distortion.

The oocyte now enters a stage when all the three categories of the sudanohil bodies (L_1, L_2, and L_3), which were hitherto easily distinguishable from e another by their size and distribution, now become intermingled and come occupy the whole of the cytoplasm uniformly. At the same time the size of e large AH-negative spheres (L_3) becomes so much reduced that it is not ossible to distinguish them from the larger AH-positive bodies (L_2) in Sudan ack B preparations. However, the granules of the first category (L_1) can still distinguished from those of the other two types (fig. 2, A, B, C). If comarison is made between oocytes in the corresponding growth periods in udan black B and acid-haematein preparations, the very much reduced umber of the larger sudanophil bodies (L_3) in the latter betrays the presence AH-negative spheres (figs. 2, A–C; 3, C–E). Intermediate, slightly AH-ositive bodies, can be made out at these stages also.

In older oocytes these AH-negative sudanophil bodies (L_3) once more ecome concentrated in the form of a cortical ring (fig. 2, C). A few of these odies are of considerable size. Since this ring is composed mostly of AH-egative bodies (L_3), it does not appear in acid-haematein preparations ig. 3, E).

With further growth of the oocyte there comes a period of great physiogical change during which all the large AH-negative (L_3) as well as larger AH-ositive bodies (L_2) gradually disappear from view. The phenomenon of isappearance starts from the nuclear region and extends ultimately to the hole of the cytoplasm. The AH-negative bodies (L_3) in the cortical ring are e last to disappear. All that remains now in the cytoplasm of the oldest

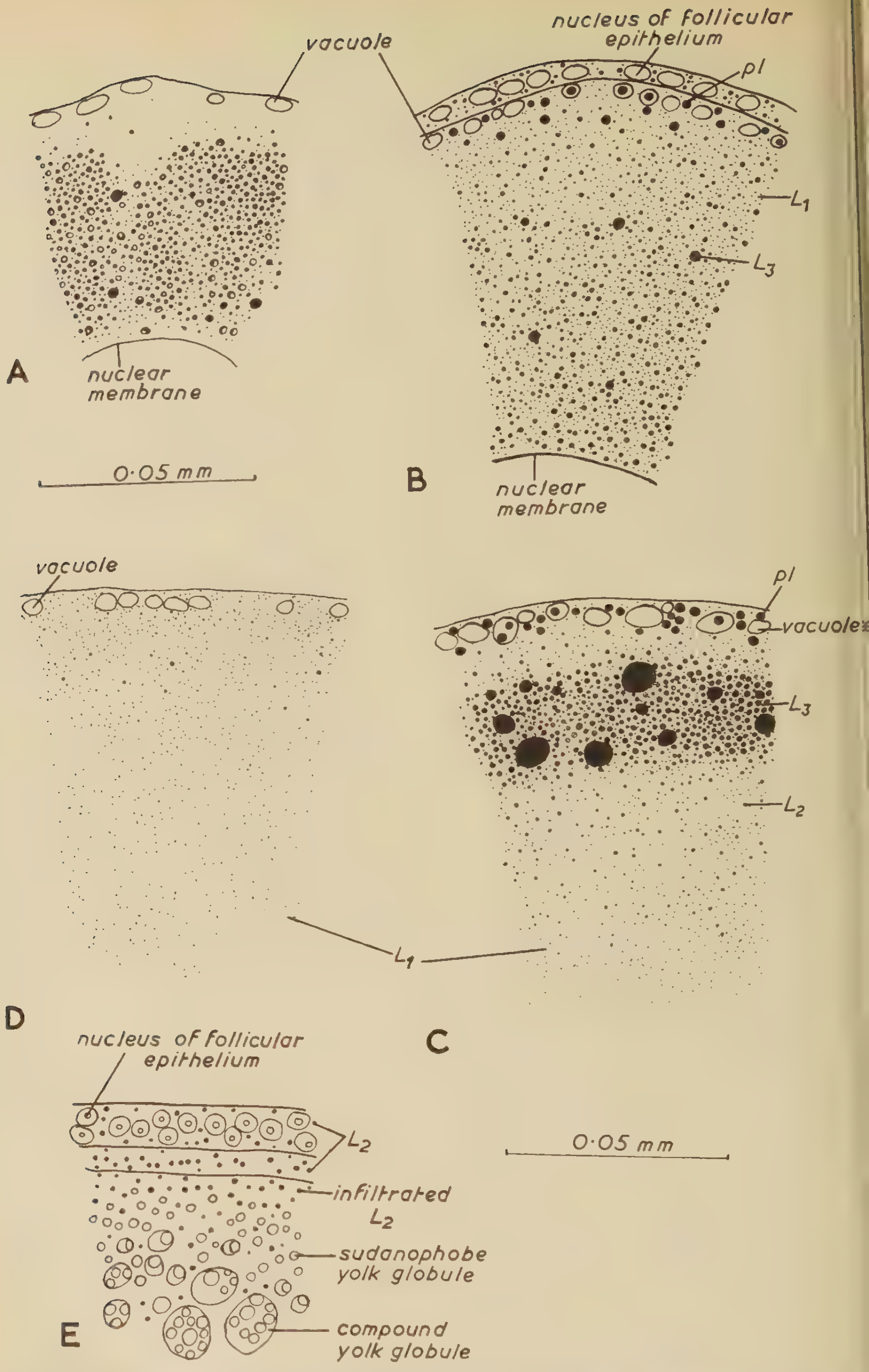


FIG. 2. A, a portion of an oocyte prepared by the Sudan black B technique of Baker (194
B and C, portions of sections of oocytes prepared by the acid-haematein technique of Ba
(1946) and coloured with Sudan black B. D, a peripheral portion of an oocyte fixed in Chan
and stained with iron haematoxylin. It shows the lipids of the 1st category only. E, a p
pheral part of the section of a ripe ovum prepared by the acid-haematein technique of Ba
(1946) and coloured with Sudan black B. It shows the infiltration of lipids of 2nd categ
through the zona radiata into the oocyte from the follicular epithelium (which is most
single layer of cells), and also the yolk globules of the periphery. L_1, L_2, L_3, lipid globules of
1st, 2nd, and 3rd categories; *pl*, peripheral lipid

ocyte examined by me are the minute sudanophil, AH-positive granules of he first category (L_1, fig. 2, D). These granules do not give pinkish tinge with Nile blue, but are stained uniformly blue with it. This means that at this late tage of growth of the oocyte the lipid granules of the first category (L_1) get ompletely shorn of their neutral fat contents. These granules can be observed n all the preparations, viz. Sudan black, acid-haematein, Nile blue, Kolatchev, Mann–Kopsch, and silver nitrate.

Once the larger sudanophil, AH-negative bodies (L_3) have disappeared nore or less completely from view, the yolk globules start appearing in the entre of the oocyte. These are at first small spherical bodies, not coloured at ll with Sudan black B, acid-haematein, Nile blue, &c.; they are completely smiophobe and argentophobe but are stained blue in Bouin / iron haematoxylin preparations. As the development of the oocyte proceeds further, hese yolk globules grow and increase in number (fig. 3, F), and correspondngly the number of minute AH-positive granules (L_1) decreases and ultinately they disappear.

With maturity the yolk globules acquire a very complex chemical nature nd start colouring with Sudan black B, and also with acid-haematein; they ppear pink with Nile blue, orange-red with Sudan III and Sudan IV, and pinkish red with Fettrot. They are also blackened by 2% osmium tetroxide olution in fresh coverslip preparations. At this time some sudanophil and AH-positive bodies belonging to the second category (L_2) can also be observed ying in the cytoplasm between the yolk globules at the periphery of the oocytes. These appear to have been infiltrated from outside the oocyte, as lipids of imilar chemical composition have been observed lying in the zona radiata and ollicle cells (fig. 2, E).

In the early oocytes, when the three types of sudanophil bodies are still in he form of a juxta-nuclear mass, some vacuoles start making their appearance n the peripheral cytoplasm (fig. 1, B). With the growth of the oocyte these vacuoles grow in number and size. At the same time some intensely AH-positive sudanophil bodies (*pl*) of comparatively large size appear inside as well as outside these vacuoles (fig. 3, B, C). The vacuoles as well as the AH-positive bodies (*pl*) increase considerably in size and in number up to a certain stage, but remain restricted to the immediate periphery of the oocyte (fig. 3, D, E). As far as histochemical reactions of the peripheral lipid bodies (*pl*) are concerned, they resemble the AH-positive sudanophil bodies of the second category (L_2), except that they are larger and are not retained in material fixed in formaldehyde-saline and coloured with Sudan black B (figs. 1, B–E; 2, A). They are clearly visible and are coloured with Sudan black B in the material fixed in formaldehyde-calcium and post-chromed, as recommended by Baker in 1946 (*pl*, fig. 2, B, C). I have not observed these bodies in material prepared by the chrome-osmium or silver nitrate techniques. These bodies as well as the vacuoles disappear from view gradually (fig. 3, F) and do not appear again. It has not been possible to discover the significance of these bodies.

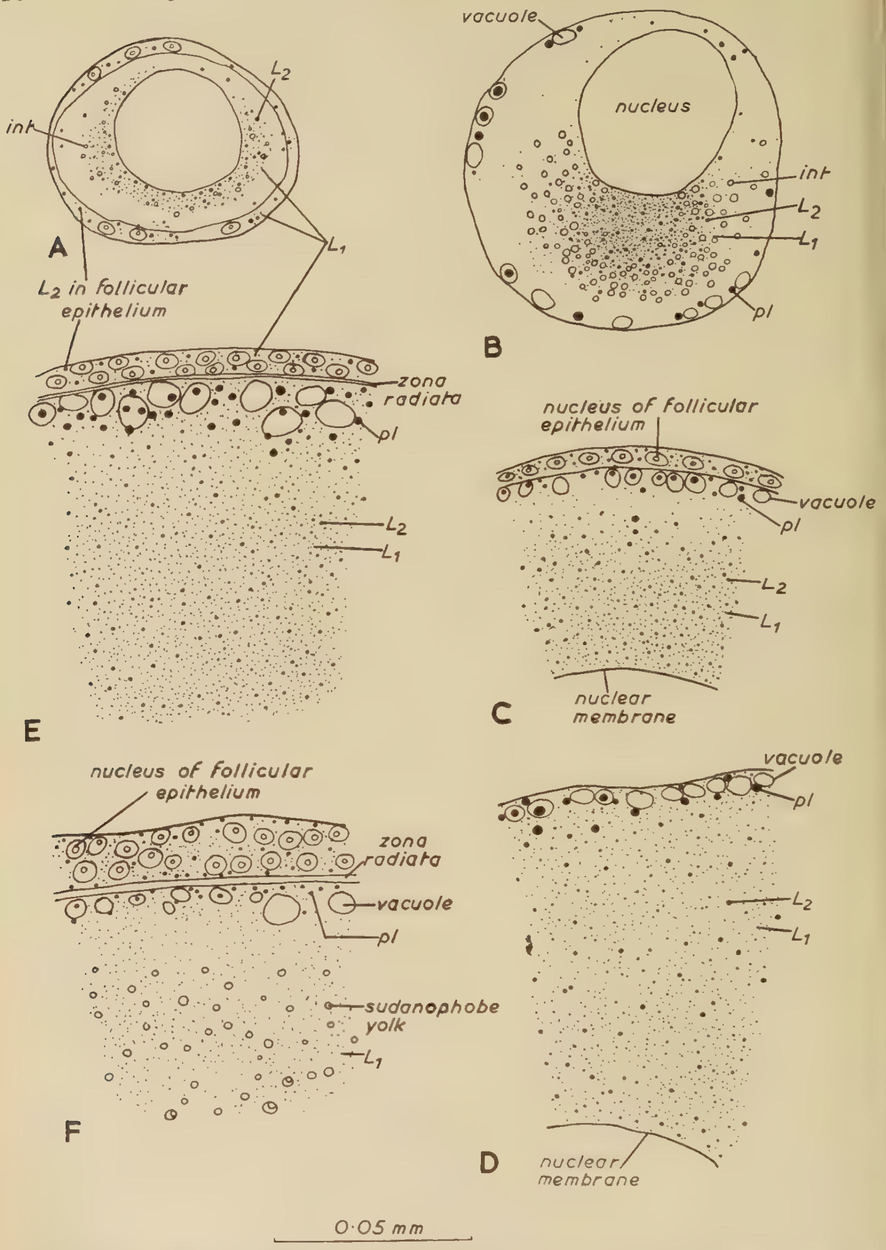


FIG. 3. A, B, sections of early oocytes prepared by the acid-haematein technique of Bake (1946). C–F, portions of sections of oocytes prepared as A, B. L_1, L_2, lipid globules of the 1s and 2nd categories; *int*, intermediate bodies; *pl*, peripheral lipid

FOLLICULAR EPITHELIUM

The follicular epithelium of the oocytes is a single layer of cells in early tages of development (figs. 1, D, E; 3, A, C). The first two categories of AH-ositive sudanophilic bodies (L_1 and L_2) described in the oocytes can also be ound in the cytoplasm of these cells. The size-difference between these two ypes of AH-positive granules exists here also.

With the growth of the oocyte, the follicular epithelium becomes multiayered (fig. 3, E, F), and there is an increase in the amount of sudanophilia orresponding with the increase in the oocyte. But when the zona radiata fully developed the follicular epithelium again becomes single-layered ig. 2, E).

SCHULTZ'S CHOLESTEROL REACTION

When Schultz's cholesterol reaction (as given by Gomori (1952)) is applied to elatine sections of material fixed in formaldehyde-calcium or formaldehyde-aline, blue-green bodies of various sizes and shapes are seen in the oocytes. he colour of these bodies fades after some time. They are soluble in acetone, cohol, and ether, and do not appear when sections are treated with these olvents. In the earliest oocytes, the cholesterol elements are present near the ucleus (fig. 4, A). With the growth of the oocyte the number of these cholesrol bodies increases, but there is a decrease in the size of the individual odies (fig. 4, B, C).

When the AH-negative sudanophil material (L_3) forms a cortical ring in the te stages, the cholesterol bodies of various sizes are seen in this region (fig. D). There is no cholesterol material in any other region of the oocyte at this age. Ultimately, however, the cholesterol material also disappears from view ong with the AH-negative spheres (L_3). Thus from their size, distribution, nd reactions it appears that cholesterol and its esters are present in the AH-egative spheres (L_3) themselves.

DISCUSSION

The histochemical use of Sudan black B has demonstrated that lipids are oundant and widely distributed in the oocytes of the pigeon. The oocyte ows a progressive increase in the amount of sudanophil lipids up to a certain age in the course of its growth. This stage is followed by disappearance most of the lipids. They reappear in the yolk globules during the late ages of yolk formation. During the early stages of oogenesis the lipids occur the form of granules and spheres of various sizes, which can be divided ughly into the following three types according to their size and histoemical reactions.

1. *Lipids of the first category* (L_1). Very minute sudanophil granules (L_1) elonging to the first category give a positive reaction with acid-haematein ntrolled by a negative reaction in pyridine-extracted material; this shows

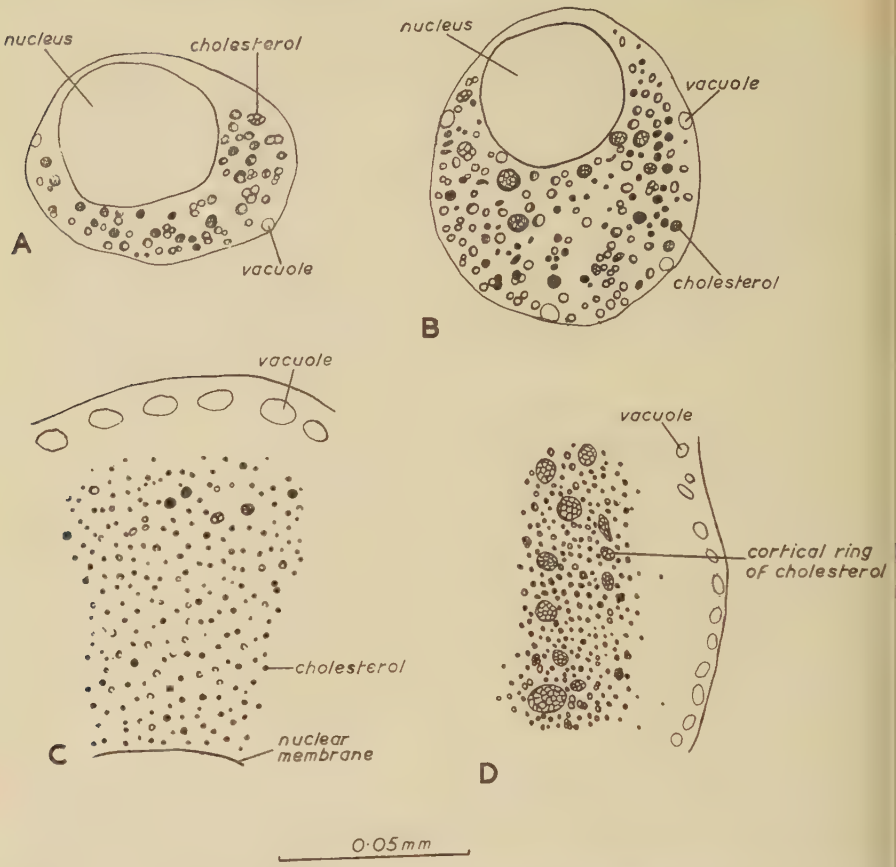


FIG. 4. A–D show the cholesterol bodies in sections of oocytes fixed in formaldehyde-saline, post-chromed as recommended by Baker (1949), and submitted to Schultz's method of cholesterol as reported by Gomori (1952). D shows the presenceof cholesterol or its esters only in the cortical region

that they contain phospholipid. With Nile blue they give a pinkish tinge; this indicates the presence of neutral fats in them. Their neutral fat content is further revealed by the fact that they colour orange-red with Sudan III and Sudan IV and pinkish red with Fettrot. Their dark blue colour with Fischler's method shows that they contain fatty acids. Besides phospholipids, neutral fats (triglycerides), and fatty acids, the presence of other material (especially proteins) cannot be ruled out, as they appear in the form of corroded granules in Bouin / iron haematoxylin preparations and in pyridine extracted material. In the latter they appear slightly yellow.

After treatment with acetone, the neutral fats (triglycerides) are lost and therefore, the intensity of colouring with Sudan black B decreases. The loss of triglycerides from these granules is confirmed by the lack of any red tinge with Nile blue and the failure of Sudan III, Sudan IV, and Fettrot to colour these granules after such treatment. But their phospholipid contents are still intact as they are coloured with acid-haematein. Their phospholipids are removed with cold alcohol and ether, as they do not colour with Sudan Black B after treatment with these fat solvents.

They resist wax-embedding after chrome-osmium fixation and are stained blue with iron haematoxylin. They might thus be said to correspond with the granular mitochondria of other workers on the oogenesis of birds. Their dark appearance in unstained Kolatchev, Mann-Kopsch, and Lewitsky preparations, studied immediately after mounting, reveals that they contain unsaturated lipid.

2. *Lipids of the second category* (L_2). The second type of sudanophil lipid bodies (L_2) are of medium size. They contain phospholipids and neutral fats, as they are stained blue-black with acid-haematein and pink with Nile blue. Their deep orange-red colour with Sudan III and Sudan IV and pinkish red with Fettrot further confirms the presence of neutral fats (triglycerides) in them. The intensity of colouring with the red Sudan colouring agents and with Fettrot depends upon the amount of triglycerides present in these lipid bodies. Thus the colour varies between the orange-red and red. These bodies differ from the minute granules of the first category (L_1), in being larger and containing comparatively more triglycerides. They also lack fatty acids and proteins, which appear to be present in the minute granules (L_1).

The lipid bodies of the second category (L_2) dissolve out completely in acetone because of the presence of triglycerides along with phospholipids. Cain (1950) and Krishna (1950) have stated that a small amount of triglycerides will cause lipids to dissolve completely in acetone. Such a possibility has also been described by Lovern (1955). Thus their solubility in acetone also suggests that they are a mixture of phospholipids and triglycerides. Here the question arises why the minute granules of the first category (L_1), which also contain phospholipids and triglycerides, do not lose their lipids completely in acetone. First, it may be possible that a thin film of triglycerides, which dominate the total sudanophilia of the oocyte, surrounds the granules of the first category (L_1). If that is so, this film is easily washed away in acetone, while the phospholipid contents remain intact. Secondly, the amount of triglycerides in these granules of the first category (L_1) may be so small that it is dissolved in acetone without affecting the presence of phospholipids. The first view is supported by the fact that with the disappearance of triglyceride spheres (L_3) from the advanced oocytes, these minute granules (L_1) also lose their triglycerides, as they no longer give a pinkish tinge with Nile blue (L_1 in figs. 2, D; 3, F).

The lipid bodies of the second category (L_2) also resist wax-embedding after chrome-osmium fixation, and they are stained blue by iron haematoxylin, but they are completely removed in Bouin / iron haematoxylin preparations. They turn jet black with silver nitrate, and in Kolatchev, Mann–Kopsch, and unstained Lewitsky preparations. Their black appearance in Kolatchev, Mann–Kopsch, and in unstained Lewitsky preparations studied immediately after mounting, shows that the lipids present in them are unsaturated. They always appear in the form of granules in these preparations. Whenever there is a collection of a few granules, the silver is deposited in the spaces between these granules and a network-like mass is produced. Thus their granular form is entirely concealed. These lipid bodies (L_2) probably correspond to the so-called 'Golgi bodies' of earlier authors on bird oogenesis.

From the observations of Nama (1956) I have not been able to understand which bodies he has called 'Golgi bodies', because he has stated that the small bodies which looked and reacted like 'Golgi elements' are not really the 'Golgi elements' but are phospholipids scattered in the cytoplasm. He further says that after treatment with alcohol and ether, the 'Golgi elements' colour with Sudan black B and he has attributed this colouring to the presence of lipoproteins in these bodies. As far as my observations are concerned the total lipid contents of the oocytes dissolve in alcohol and ether, except a mass of sudanophil bodies observed in the juxta-nuclear area at a particular stage of the oogenesis only (fig. 1, H, *lip*). This mass of sudanophil material loses its sudanophil nature when the material is treated with boiling alcohol only. This mass can be interpreted, by the use of selective staining and solubility tests (Krishna, 1950, 1953; Nama, 1956), as lipoprotein bodies, which, owing to the vigorous action of fat solvents, have formed one compact mass. It is, therefore, probable that Nama has described this juxta-nuclear mass of lipoproteins as the 'Golgi bodies'. These lipoprotein bodies appear to be separate from other lipid bodies (L_1, L_2, and L_3).

3. *Lipids of the third category* (L_3). The third type of lipid bodies (L_3) are large, intensely sudanophil spheres which remain completely negative after the AH-test. Their reactions suggest the absence of phospholipids, which are present in the first two categories of lipid bodies (L_1 and L_2). Their deep pink colour with Nile blue shows that they are droplets of neutral fats. Their red coloration with Sudan III and Sudan IV and deep pinkish red colour with Fettrot further reveal that they are made up of triglycerides. Their triglyceride nature is also confirmed by their solubility in acetone. They are not preserved in Bouin / iron haematoxylin preparations. Their black colour in unstained Lewitsky preparations, studied immediately after mounting, indicates the unsaturated nature of the triglycerides of these bodies (L_3).

It has already been pointed out in observations that these lipid bodies (L_3) give a positive reaction with Schultz's method after oxidation in iron alum, but not without it. Thus, besides triglycerides, cholesterol or its esters also form a part of their fatty contents. Marza and Marza (1936) have also found that the fatty balls of early oocytes of the hen and the 'cortical' granular layer of fat in the advanced oocytes are rich in cholesterol.

Singh (1938) and Nama (1956) have not been able to state clearly whether cholesterol exists in separate globules or is present in the neutral fat globules. They have simply mentioned the presence of cholesterol in the oocytes of the pigeon. The various forms and shapes of the cholesterol bodies described by Singh (1938) can be attributed first to the drying effect on the neutral fat bodies and secondly to the quick dehydration by the acids used in the Schultz's reaction.

Although it is rather difficult to correlate the various lipid bodies of the pigeon oocytes with each other, one is tempted to conclude that the larger neutral fat bodies (L_3) arise by the growth and chemical change of the lipid bodies of medium size (L_2). This conclusion is strengthened by the fact that intermediate forms (figs. 3, A, B; 1, G) of various sizes, with a chemical composition varying between these two, occur quite commonly.

The differential staining of these intermediate bodies (*int*) with acid-haematein suggests that the medullary region contains neutral fats (triglycerides), as it is negative to AH-test, while the cortical region contains triglycerides masked with phospholipids, as it gives a feeble positive reaction with acid-haematein. As these bodies grow, the rim is attenuated with the result that the whole of the sphere becomes AH-negative. Thus it appears that with growth in size the phospholipid contents of the large AH-positive bodies (L_2) are either transformed into neutral fats (triglycerides) or disappear from these bodies. The duplex structure of the intermediate bodies (*int*) in the material prepared by the Kolatchev and silver nitrate methods, and in the post-chromed material fixed in formaldehyde-calcium and treated with acetone, alcohol, and ether, can be explained by the fact that the triglycerides of the central part are dissolved by the fat solvents, while the fixed phospholipids of the rim resist the action of these fat solvents.

This growth and chemical transformation of the large AH-positive bodies (L_2) (the so-called 'Golgi bodies') into AH-negative spheres (L_3) (the so-called 'fatty yolk' of earlier authors) appears to be exactly like the origin of 'fatty yolk' from the 'Golgi vesicles' as described by Nath since 1929. References may be made to Nath (1957) for full bibliographies on this subject.

It also appears that the larger AH-positive bodies (L_2, the 'Golgi bodies' of other authors) arise from the smallest AH-positive granules (L_1, the 'mitochondria' of other authors), as suggested by Hirsch (1939). Reference may also be made to Nath (1956, 1957) for full references on the subject.

4. *Yolk globules.* Before the appearance of yolk globules nearly all the lipids disappear from view except some small neutral fat granules in the cortical region (L_3), the minute AH-positive granules (L_1), and the peripheral lipids (*pl*).

The yolk globules, when they first appear, are completely sudanophobe, but as they grow in size they correspondingly attain an appreciable amount of sudanophilia, which is diffused throughout the globule. This sudanophilia gives positive reaction with the AH-test and a negative reaction with PE, which indicates the presence of phospholipids. Their pinkish coloration with Nile blue, orange-red with Sudan III and Sudan IV, and pinkish red with Fettrot, shows that triglycerides are also present in the yolk globules. The lipid nature of these yolk globules is further revealed by their going black quickly with 2% osmium tetroxide solution in fresh coverslip preparations. This shows that the lipids present in these yolk bodies are unsaturated. All these reactions show their compound lipid nature with phospholipids and triglycerides present. That proteins or carbohydrates or both may be present is suggested by the fact that they resist pyridine extraction and stain blue with iron haematoxylin after Bouin. A similar type of compound yolk globules has also been described by Marza and Marza (1936) in the hen's egg. They found that the transitional forms between the primordial yolk (my sudanophobe yolk globules) and the yellow yolk of the ripe egg contain various quantities of neutral fats and lipids.

5. *Peripheral lipid bodies* (*pl*). From the colour tests and solubility tests it appears that the lipid bodies (*pl*) found in connexion with the vacuoles of the periphery are of more or less the same chemical composition as the larger AH-positive bodies (L_2), i.e. they contain phospholipids and some neutral fat (triglyceride), but they are larger than the latter.

These peripheral lipid bodies (*pl*) differ from the larger AH-positive bodies (L_2) as they are not preserved in material fixed in formaldehyde-saline. They also do not show well in material fixed in formaldehyde-calcium without post-chroming. They appear as intensely sudanophil bodies in the sections fixed and post-chromed by the acid-haematein technique of Baker (1946). This thus indicates the different chemical nature of the phospholipids of these bodies from those of the second category (L_2). It appears that the calcium ions in the preliminary fixation and the post-chroming with calcium-dichromate are necessary for the retention of the phospholipids of these bodies. This observation is in conformity with the view expressed by Cain (1950) and Baker (1956). Similar observations of the use of calcium ions in the preliminary fixation have also been made by Gupta (unpublished) in this laboratory on the male germ-cells of ticks.

I wish to express here my deep sense of gratitude to Professor Vishwa Nath for suggesting to me this line of research, for his keen interest and constant encouragement throughout the progress of this work, and also for correcting the manuscript for the press; to the Editors of this Journal for sending me valuable suggestions and criticisms; to B. L. Gupta for helpful discussion; and to the University Grants Commission and the Panjab University for the financial help to carry out the work.

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The Effect of Acetic Acid on Cytoplasmic Inclusions

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With one plate (fig. 1)

SUMMARY

Acetic acid at 5% is not necessarily destructive of cytoplasmic inclusions. Hermann's fluid gives excellent mitochondrial preparations if tissues are postosmicated for several days at 34° C. The mitochondria are blackened by this treatment. Mann's fluid with the addition of 5% of acetic acid gives, on postosmication, very good preparations of the 'Golgi apparatus' of certain cells.

INTRODUCTION

IT was remarked by von Brunn in 1884 that the granules grouped round the axial filament of the mammalian spermatid 'show the reaction of protoplasmic granules on treatment with acetic acid; that is to say, they first become pale, then indistinct, and finally disappear altogether, while the axial filament remains still clearly recognizable'.

It was this finding of von Brunn's that led Benda (1902) to reduce greatly the amount of acetic acid when making up Flemming's strong fluid for studies of mitochondria, and this example was followed by others. Acetic acid was omitted from Flemming's fluid by Lewitsky (1911) in his studies of mitochondria. No acetic acid was included by Golgi (1898) in the fixative he used to demonstrate his 'apparatus'. It has always been omitted or used at low concentration by his successors.

As a result of the example of these and other authorities, the general practice is to omit acetic acid from fixatives used in studies of cytoplasmic inclusions, or to reduce its concentration far below 5%. In general histology and studies of chromosomes, on the contrary, it is usual to include acetic acid at 5% v/v or thereabouts, because its swelling effect counteracts the shrinkage caused by other constituents of fixative mixtures.

Two questions are raised by these familiar practices. Is it possible that acetic acid at about 5% does not in fact necessarily destroy mitochondria? And if it does destroy them, *how* does it do so?

With regard to the first question, those who have placed living cells in solutions of acetic acid have not always observed a sudden destruction of mitochondria (Lewis and Lewis, 1915; Strangeways and Canti, 1927), though filamentous ones are often transformed into faint rows of granules. Chromium trioxide, used alone, is much more destructive than acetic acid; yet it is included in several valuable fixative mixtures used in studies of mitochondria. These organelles (or the remains of them) have been seen from time to time in microscopical preparations made from tissues fixed in solutions containing quite high concentrations of acetic acid (Champy, 1911; Romeis, 1913;

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Nicholson, 1916; Young, 1928; Baker, 1932; Marquez, 1934). Neverthele there can be no doubt that as a general rule one does not obtain a clear vie of them if acetic acid is used at the concentration that is usual in routi histology.

With regard to the second question, it is pointed out that mitochondria ar certain other cytoplasmic inclusions are composed partly of lipids, and th acetic acid is a lipid-solvent. This, however, is irrelevant; for although glaci acetic acid does indeed dissolve certain lipids, the aqueous solution at 5% do not. The hydronium ion cannot be responsible, for, as Casselman and Jorda (1954) showed, one can actually fix mitochondria in 0·1 N hydrochloric aci There is no obvious reason why the acetate ion or the undissociated acid, aqueous solution, should disperse lipids.

The known facts, taken together, lead to the following conclusions:

(1) Acetic acid, used by itself at 5%, is not necessarily very destructive mitochondria.

(2) It is not, however, a fixative for mitochondria: that is to say, it does n protect them against dehydration and other processes of routine micr technique.

(3) There is no proof that acetic acid at 5% might not be used as a co stituent of a fixative mixture intended for use in studies of mitochondri provided that steps were taken to render the mitochondria insolub in fluids that would have to be used subsequently.

Osmium tetroxide is a reliable fixative for mitochondria, if allowed to a for sufficient time. It was therefore decided to try various fixative mixtur containing acetic acid at 5%, and subsequently to treat the tissues wi osmium tetroxide solution before dehydrating and embedding them.

Material and Methods

Most of the work has been done with the epididymis, pancreas, and anteri part of the intestine of the mouse. A wide range of tissues from mamma urodeles, and various invertebrate groups has also been studied.

The following fixatives were used: Zenker's fluid (1894); Flemming strong fluid (1884) with full acetic; Hermann's fluid (1889, the fluid design for mammalian tissues); acetic-osmium (Hermann's fluid without chlor platinic acid); and Mann's mercuric-osmium (1894), with the addition acetic acid at 5%. All these fixatives contain acetic acid at exactly or almo exactly 5% v/v. The last three are conveniently made up in accordance wi the table on p. 427.

Pieces of tissue about 2 mm or less in thickness were used. The strand co necting the caput with the cauda of the epididymis was used, because it is conveniently narrow. It was found best to leave a short piece of the intesti in the fixative for 5 min before opening it longitudinally. If this organ opened before it is put in the fixative, it tends to turn inside out and the vi are then likely to be damaged during dehydration or embedding.

The pieces were fixed for 24 h. They were then washed in water and tran ferred to 2% osmium tetroxide solution. The period of washing, the period in the osmium tetroxide solution, and the temperature of osmication were varied. After osmication the pieces were washed for a few hours in changes of distilled water and then passed either through graded ethanols and toluene into paraffin or else through cellosolve into estax (Chesterman and Leach, 1956).

	Hermann's fluid (mammalian formula)	*Acetic-osmium*	*Mann's fluid plus acetic acid*
	(ml)	(ml)	(ml)
Distilled water . .	0·8	1·1	. .
Chloroplatinic acid, 5% aq.	0·3	. .	. .
Osmium tetroxide, 2% aq.	0·4	0·4	0·5
Acetic acid, 20% aq. .	0·5	0·5	0·5
Mercuric chloride, sat. sol. in 0·75% NaCl . .	. .	. .	1·0
	2·0	2·0	2·0

Sections were generally cut at 5 μ and mounted in Canada balsam or DPX. Dyeing was unnecessary, since the cytoplasmic inclusions, if present, could be seen as grey or black objects.

RESULTS

Mitochondria were usually visible, at any rate in some part of the piece of tissue, and they were often blackened if the period and temperature of osmication had been right for this purpose. They were often changed from filaments or rods into spheres, however, and in some cases the ground cytoplasm was darkened, so that they did not show very clearly. Satisfactory results were not obtained when Zenker's or Flemming's fluid was used as fixative.

Remarkably clear preparations of mitochondria were obtained when Hermann's fluid was used as fixative. It was found best to wash the pieces of tissue thoroughly after fixation. Short or careless washing resulted in poor osmication. Running water should be used, but since various ions interfere with the reduction of osmium tetroxide, it is a good plan to give a final wash in distilled water, though this is not necessary if the tap-water is reasonably pure. The osmium tetroxide must be warm if the mitochondria are to be blackened. At room temperature even 36 days' osmication does not blacken them, though some of them become pale grey. Less good results are obtained at 37° C than at 34°. At the higher temperature the cytoplasm usually becomes grey, so that the mitochondria do not stand out nearly so clearly as when the slightly cooler fluid is used. It is curious that such a small difference in temperature should have such a marked effect on the result. Six days' osmication suffices to give black mitochondria; this period may sometimes be halved. If pieces of tissue of the proper size be used, one may osmicate with only 0·5 ml of solution. If the fluid becomes grey or yellowish during osmication, it must be replaced.

The procedure may be summarized thus:

(1) Fix for 24 h in Hermann's fluid (with full acetic).
(2) Wash thoroughly in running water for 24 h. Give a final wash in distilled water.
(3) Leave in 2% osmium tetroxide at 34° C (in a small, tightly closed specimen-tube in the dark) for 3 to 6 days. (It is best to try 6 days first.)
(4) Wash for several hours in changes of distilled water.
(5) Embed in paraffin or estax.
(6) Cut sections at 5 μ (or less).
(7) Mount in Canada balsam or DPX.

Since the essential parts of this method are fixation in Hermann's fluid and postosmication, it may be called the Hermann PO or HPO technique. It gives very clear preparations of mitochondria, which appear in black against a nearly white background; chromatin is just touched with yellow from the chloroplatinic acid. Mitochondria are not shortened or broken up into rows of globules by this method, but they are sometimes considerably thickened. This is the easiest and most certain of all ways of making permanent preparations of mitochondria. The pancreas (fig. 1, A) and intestine of the mouse are among the easiest tissues from which to make striking preparations. The method usually succeeds with cells in which the mitochondria are rather difficult to show by the usual methods (the nerve-cells of mammals, for instance). It fails, however, with the nerve-cells of the cricket (*Acheta domesticus*), which are badly fixed, strangely enough, by Hermann's fluid. The large mitochondria of the first convoluted tubules of the mammalian kidney are not very well shown.

If the acetic acid is reduced to 2·5% or omitted, the result is not nearly so good. The acetic acid opposes the tendency of the chloroplatinic acid to shrink the cells.

If the chloroplatinic acid is omitted from Hermann's solution (or, to put it in another way, if acetic-osmium is substituted for Hermann), the result is entirely different, though the whole of the rest of the technique remains unaltered. The mitochondria are seen only vaguely or not at all, and the cytoplasm is grey. It is to be presumed that chloroplatinic acid acts upon lipoprotein complexes in such a way as to separate or 'unmask' the lipid constituents, and that these are fixed and blackened by osmium tetroxide.

Some of the various objects commonly called 'Golgi apparatus' are also blackened by the HPO technique. This applies to the material surrounding the developing secretion-globules in the epithelial cells of the epididymis and in the Paneth cells of the crypts of Lieberkühn of mammals. The so-called Golgi apparatus of the intestinal epithelium of mammals is evidently of a different nature, for it remains invisible by this technique. It may be recollected that Kolatchev (1916), in his studies of the 'Golgi apparatus' in the

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FIG. 1 (plate). A, pancreas of mouse. Hermann's fluid; postosmicated for 6 days at 34° C. B, epididymis of mouse. Mann's fluid with the addition of 5% of acetic acid; postosmicated for 3 days at 34° C.

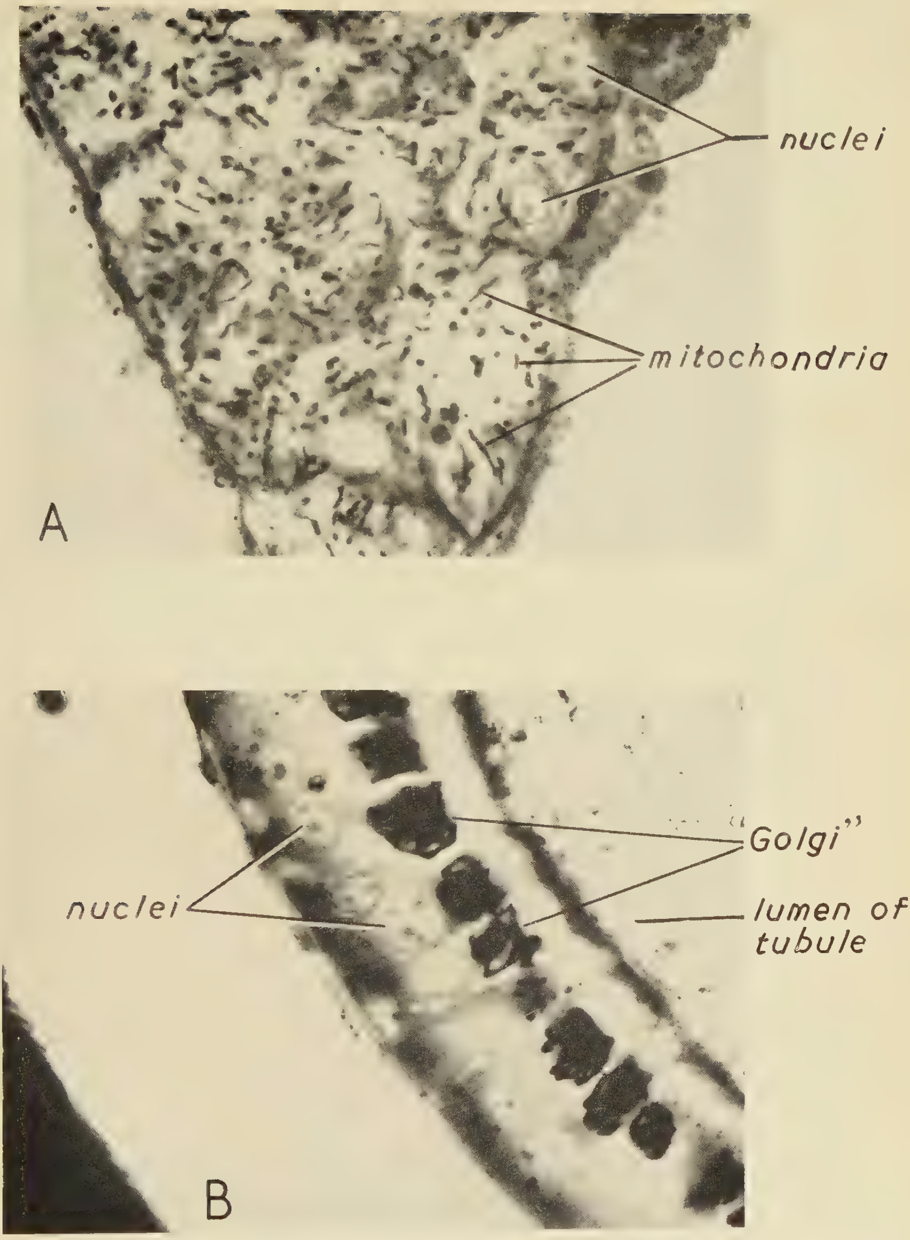

Fig. 1

J. R. BAKER

neurones of gastropods, sometimes fixed in a modified Hermann's fluid and postosmicated. In his fixative, however, the concentration of acetic acid was greatly reduced.

When chloroplatinic acid is omitted from Hermann's solution, the 'Golgi apparatus' is still blackened in the epididymis. It is evident that no unmasking process is necessary in this case. Indeed, the material blackened is probably not lipid (see Christie, 1955). Such preparations are poor, however, because the cytoplasm is grey.

If Mann's mercuric-osmium, with the addition of 5% of acetic acid, be substituted for Hermann's, it is best to reduce the period of osmication at 34° C to 3 days, in order to avoid darkening of the ground cytoplasm. The change of fixative profoundly modifies the picture. Mitochondria are now poorly shown in most tissues, but some of the various objects to which Golgi's name is usually attached are shown with astonishing clarity. The epithelial cells of the epididymis of the mouse provide a good example (fig. 1, B). The intestinal epithelium, on the contrary, reacts in rather a patchy way. A fairly characteristic picture of the Golgi apparatus is seen in some of the cells of the anterior mesenteric ganglion of the rabbit, but it appears to be made up mostly of separate threads. It is possible that these are in reality thickened mitochondria. It may be recalled that several authors, especially Monti (1915), have claimed that the Golgi apparatus of the neurone of vertebrates actually represents the mitochondria.

Mrs. B. M. Jordan-Luke has given me a great deal of practical help in this work. Mr. S. Bradbury and Mr. J. T. Y. Chou have kindly tried out the HPO technique on various tissues and given me the benefit of their experiences.

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The Fixation of Adipose Fat by Potassium Dichromate

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SUMMARY

Ordinary adipose fat globules can be rendered resistant to paraffin embedding by the immersion of suitably fixed tissues in a saturated solution of potassium dichromate at 37° C for 7 weeks. Xylene has less tendency to remove the postchromed fat than chloroform, benzene, or toluene.

Adipose fat is usually dissolved out of the tissues during dehydration or embedding. Empty spaces are therefore usually seen in paraffin sections wherever fat globules were present during life. Several authors (Kaufmann and Lehmann, 1926 *a*, *b*; 1928; Smith, Mair, and Thorpe, 1909) have claimed that potassium dichromate can in certain circumstances be used to fix adipose fat in such a way that it is retained in paraffin sections, but a full study of this subject has not previously been made. My object has been to find a reliable method of fixing adipose fat by potassium dichromate in such a form that it is not dissolved away when paraffin sections are made.

MATERIAL AND METHODS

THE skin of the mouse was used for this investigation as the subcutaneous adipose cells are large and abundant. It was shaved with a razor-blade and cut into pieces about 1 cm square.

The skin was always fixed for 2 days in Ciaccio's fluid (formalin 20 ml; potassium dichromate, 5% aq., 80 ml, glacial acetic acid, 5 ml). This was chosen because it was known not to be destructive of lipids. After washing in running water for 24 h, the skin was soaked in 3% potassium dichromate for 24 h at room temperature, and then transferred to a saturated solution of potassium dichromate in an incubator at 37° C. The period in the saturated solution varied from 1 to 7 weeks. The intention was to discover whether potassium dichromate would render adipose fat insoluble in the fluids used for dehydration and embedding, and if so, what period of postchroming was necessary.

A standardized process of dehydration was used throughout. After the tissue had been washed for 24 h in running water it was left for ½ h in each of the following grades of ethanol: 50%, 70%, 80%, 90%, 95%, and absolute.

Four different antemedia were tried: namely, chloroform, benzene, toluene, and xylene. The intention was to discover which of these had the least tendency to dissolve the fixed adipose fat. The piece of tissue was first left for ½ h in a mixture of equal volumes of absolute alcohol and antemedium, and then for ½ h in the antemedium alone. The piece was then transferred to melted paraffin wax (m.p. 56° C) and left for 1 h (with one change of wax).

Sections were cut at 15 μ and were coloured for 5 min in a saturated solution of Sudan black in 70% ethanol. They were rinsed for 5 sec in 70% ethanol

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and 1 min in 50%; then they were washed in distilled water and mounted in Farrants's medium.

RESULTS

Different degrees of preservation of the fat were shown in different preparations. In many cases no sudanophil material survived dehydration and embedding. When a little remained, it usually appeared in the form of a network extending across an otherwise empty space. When a considerable amount remained, it was mainly aggregated at the periphery of the globule, while the centre either contained a network of sudanophil material or else was empty. (The partial solution of fat globules was studied long ago by Starke (1895), who showed that the undissolved remnant formed a rim round an empty space.) When the whole of the globule was preserved, it appeared homogeneously black. In some cases the globules were well preserved near the edge of the piece of tissue, but imperfectly towards the centre.

The various degrees of preservation of the fat are shown by symbols in table 1. The symbols are explained in the legend to the table.

TABLE 1

The effect of different periods of postchroming and of different antemedia on the preservation of adipose fat

Period of postchroming at 37° C (weeks)	*Antemedia*			
	Chloroform	*Benzene*	*Toluene*	*Xylene*
1	o	o	o	o
2	o	o	o	o
3	o	o	o	o
4	+	+	+	+
5	++	++	++	++
6	++	++	++	+++
7	+++	++	++	++++

++++ Full coloration by Sudan black.

+++ Fat globules near the edge of the section were solid black, while those near the centre of the section showed a network.

++ The margins of the globule were solid black, but the interior showed a network.

+ means that only a network of sudanophil material was left.

o No sudanophil material was present.

The table shows that no adipose fat is preserved unless the period of postchroming exceeds 3 weeks, whichever antemedium is used. With longer periods of postchroming the preservation is progressively improved. Of the various antemedia, xylene has the least tendency to extract the fat, and benzene and toluene the most, chloroform is intermediate. If postchroming is continued for 7 weeks, and xylene is used as an antemedium, the fat droplets appear homogeneously black in Sudan black preparations.

DISCUSSION

It was discovered by Smith, Mair, and Thorpe (1909) that triolein can be rendered insoluble in ethanol, xylene, and ether by prolonged postchroming.

Kaufmann and Lehmann (1926 *a*, *b*; 1928) claimed that all unsaturated lipids could be rendered insoluble in lipid solvents by the action of potassium dichromate, different lipids requiring different periods. The preservation of various conjugated lipids by short postchroming is a familiar process of microtechnique. The facts presented in the present paper show that ordinary adipose fat can be successfully postchromed, but the period has to be very long. The postchromed fat is less soluble in xylene than in other antemedia commonly used in paraffin embedding.

I wish to express my gratitude to Dr. J. R. Baker for suggesting and supervising this investigation, and also for helping in correcting this paper; to Professor Sir Alister Hardy, F.R.S., for providing me with facilities for working in his Department.

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The Size of Living Bacteria

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With one plate (fig. 4)

SUMMARY

The difficulties involved in the direct measurement, by eyepiece-micrometer or from photomicrographs, of small microscopic objects such as living bacteria are discussed. The accuracy with which this can be done is limited by the numerical aperture of the optical system and the wavelength of light used. With visible light it is scarcely possible to determine the dimensions of an object more accurately than to the nearest 0·4 μ. It also seems probable, from the nature of the diffraction pattern at the edges of images of objects of circular cross-section such as bacteria, that direct measurement of the width of the image will tend to give an underestimate of the true width of the object.

An interference microscope enables thickness measurements to be made that are not subject to these particular limitations, because with it, the phase-change in the light passing through the middle of a bacterium can be measured very accurately. This phase-change is proportional to the product of the refractive index of the bacterium minus that of the mounting medium, and its true thickness.

Two methods were used to determine the mean thickness of the living bacilli in a number of different cultures of *Lactobacillus bulgaricus*. With the first, the mean refractive index of the bacilli was measured directly by the method of immersion refractometry first used by Barer and Ross (1952), and phase-change measurements were made on the bacilli mounted in dilute saline. Their mean thickness was calculated from these measurements. With the second method, phase-change measurements were made on the bacilli mounted in saline and also mounted in protein solutions with refractive indices ranging from 1·365 to 1·376; and, from these, both their mean thickness and their mean refractive index were calculated.

The phase-change measurements were made with a Smith interference microscope and half-shade eyepiece (manufactured by Messrs. Charles Baker).

The values for the mean thickness of the living *L. bulgaricus* from 14 different cultures obtained by the first method ranged from 1·13 μ to 1·23 μ; and those from 9 different cultures obtained by the second method ranged from 1·02 μ to 1·14 μ. The mean refractive indices of the latter calculated by the second method agreed very closely with that obtained by immersion refractometry, and differed by a maximum of 0·009 in all the cultures measured. It therefore seems unlikely that the mean thickness measurements obtained by either method are wrong by more than about ±0·1 μ.

INTRODUCTION

Direct measurement of small microscopic objects

THE dimensions of microscopical objects are normally measured in the plane at right angles to the optical axis of the microscope by means of a calibrated scale in the eyepiece. The accuracy with which this can be done is limited by the numerical aperture of the optical system and the wavelength of light used. Microscopes using visible light with objectives of the highest attainable numerical aperture (1·4) will not resolve details that are closer

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than about 0·25 μ apart as separate points. Consequently, it is found that the edges of microscopic objects magnified by more than about 300 times never appear as sharp lines, but will always look blurred, and show boundary gradients of varying intensity due to diffraction.

The intensity gradients across a uniformly light-absorbing object of square cross-section of the order of size of a living bacterium, seen in visible light with a 2-mm objective, will approximate fairly closely to the curve shown in fig. 1, A. C and C' represent the actual position of the edges of the square cross-section of side t. The main intensity gradients are in the zones between A and B, and A' and B'. Outside AA' a number of minor fluctuations (Airy patterns) may be visible, but the largest of these, R, will have a maximum amplitude of only one-fifty-seventh of that of the main gradients if there is no spherical aberration in the lenses of the optical system (Airy, 1835); and its amplitude will certainly not exceed one-twenty-fifth of that of the main gradients in most ordinary microscopes (Martin, 1926).

The width of the main gradient of intensity-change at each boundary h can be determined by the formula:

$$h = \frac{0{\cdot}61\lambda}{N}, \tag{1}$$

where λ = the mean wavelength of the light used and N = the numerical aperture of the objective and condenser. The actual boundaries of the correct image of *any* microscopic object will always lie somewhere within the limits of this gradient; and, in the present case of an object with a square cross-section, they will be at C and C', exactly midway between A and B, and A' and B' respectively.

Thus, the true width of an object of square cross-section (lying with two of its sides parallel to the optical axis of the microscope) may be arrived at by measuring the image from the mid-points of the main diffraction gradients at its edges. Alternatively, it may be determined from the formula:

$$t = w - \frac{0{\cdot}61\lambda}{N}, \tag{2}$$

where t = the true width of the object and w = the maximum width of the main diffraction gradient round the image (= the length AA' in fig. 1).

This is mainly of theoretical interest, because very few microscopic objects have a square cross-section. There are, however, many biological objects of microscopic size with a circular cross-section; and these include a great many kinds of living bacteria. The exact form of the intensity gradients across an object of circular cross-section, such as a cylindrical bacillus, is very difficult to determine exactly, but they will have a less steep slope than the curves shown in fig. 1, A, and will be, very approximately, of the shape shown in fig. 1, B. It is immediately obvious that a measurement between the mid-points of these shallower gradients (the length DD' in fig. 1, B) will *not* indicate the true width t of the cylinder, but will indicate a rather *lesser*

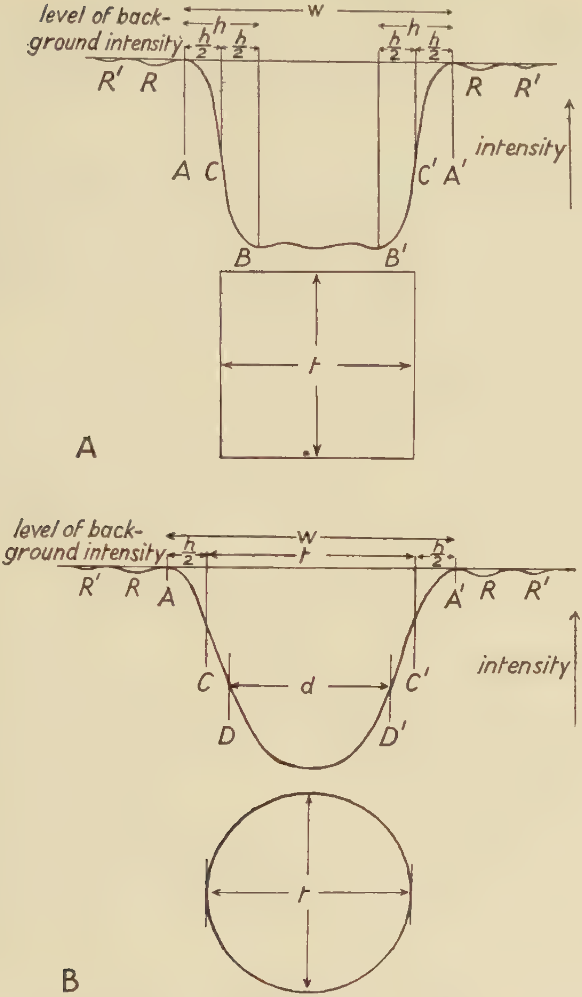

FIG. 1. A, the approximate distributions of intensity across the image, under a 2-mm microscope objective, of a uniformly light-absorbing microscopic object of square cross-section with a side *t* (shown below), of the size of a living bacterium. Ordinate = intensity. Abscissa = linear distance across the object at right angles to the optical axis of the microscope. For full explanation see text. B, the approximate distributions of intensity across an image, under a 2-mm microscope objective, of a uniformly light-absorbing microscopic object of circular cross-section with a diameter *t* (shown below), of the size of a living bacterium. Ordinate = intensity. Abscissa = linear distance across the object at right angles to the optical axis of the microscope. For full explanation see text

width (*d*). This effect will be even more pronounced if the object is not a cylinder but an ellipsoid, or a sphere as in the case of a coccus, because in these cases the gradients due to diffraction would be even less steep than in the case of the cylinder.

From this, because it is usual for an observer to estimate the width of a microscopic object from approximately the mid-points of the diffraction gradients at its edges, one is led to an important conclusion: microscopic objects of circular cross-section will tend to *appear* rather smaller than they really are.

It should be noted, however, that, in theory, formula (2) above should be just as applicable to objects of circular cross-section as to objects of square cross-section; so it should be possible to determine their true width by measuring the maximum width (w) of the main diffraction gradients at the edges (the distances AA' in fig. 1, A, B). In practice, it is often very difficult to determine these outer limits (A and A') very precisely, particularly when the gradients themselves are not very steep.

It must also be emphasized that the above arguments are only strictly applicable in the case of uniformly light-absorbing objects seen under ordinary conditions, and with a condenser that has a numerical aperture the same as that of the objective. They are not necessarily applicable to phase contrast or dark ground images, where the diffraction patterns may be appreciably modified.

From formula (1) above, it can be seen that, even if one uses an objective of the highest obtainable numerical aperture (1·4) and makes measurements in the shorter wavelengths of the visible spectrum (e.g. in blue/violet light with a wavelength of 470 mμ), the width of the main diffraction gradient at each edge of an object of square cross-section (h) will be about 0·2 μ; and it has already been shown that these gradients will be even wider than this round objects of circular cross-section.

One may therefore conclude that, if visible light is used, it is scarcely possible to determine the dimensions of a microscopic object accurately to more than the nearest 0·4 μ by direct measurement. Therefore the error in measuring an object of the shape and size of a living bacterium may be quite considerable.

Size derived from phase-change measurements

Living bacteria and other cells mounted in water or saline do not usually absorb light to any appreciable extent, and so do not, in this respect, behave like the microscopic objects already discussed. The light passing through them, however, is almost always appreciably retarded in phase compared to that passing through adjacent regions of the mounting medium. An interference microscope can show up these phase-changes in the form of intensity differences. It can therefore make such an object appear very much as if it were light-absorbing; but not exactly so, because the relationship between phase-change and intensity is only linear over small ranges (Mitchison, Passano and Smith, 1956).

However, an interference microscope also enables these phase-changes to be actually measured, and with considerable accuracy. These phase-changes are in the light travelling in the direction of the optical axis of the microscope. It therefore follows that the phase-retardation measured through the centre or mid-line of a bacterium will be *directly* proportional to the true thickness of the organism, and (provided the organism is large enough to be resolved) this will be independent of any intensity gradients due to diffraction at the edges of the image.

The phase-change in light passing through the middle of a bacterium will be proportional to the product of the refractive index of the bacterium minus that of the mounting medium, and its true thickness. Consequently, the true thickness can be deduced from phase-change and refractive index measurements, and the accuracy with which these can be made will determine the accuracy of the values obtained for the thickness.

The refractive indices of living bacteria can now be measured accurately to the nearest 0·001, or more accurately than this, by the method of immersion refractometry first used by Barer and Ross (1952); and the Smith interference microscope (manufactured by Messrs. Charles Baker of Holborn) is capable of measuring phase-changes to the nearest seventieth of a wavelength, or often even more accurately.

Consequently, it was decided to use this method in order to try to obtain a more accurate estimate than hitherto of the mean thickness of a living bacillus.

MATERIAL

The organism measured, a typical strain of *L. bulgaricus*, was obtained in milk cultures supplied by a commercial yoghourt firm (Les Laboratoires 'Yalacta', 51 Rue Lepic, Paris), where it is grown under very constant conditions. With the light-microscope the living (rod-shaped, cylindrical) bacilli always appeared to be between 1 μ and 1·4 μ in diameter; and eyepiece micrometer measurements showed no detectable variations in the thickness of the bacilli in cultures supplied at different dates, or in the subcultures that were grown from them in sterilized milk. As well as this single species and strain of *Bacillus*, the cultures also contained small numbers of two other organisms, a strain of *Streptococcus* and an encapsulated *Diplococcus* (*s* and *d* in fig. 4, C). They were easily distinguishable from the *L. bulgaricus*, which was the only organism on which measurements were made.

METHODS AND MEASUREMENTS

Two main methods were used for measuring the mean thickness of the living *Lactobacillus bulgaricus*.

With the first method, the mean refractive index of the bacilli was measured by immersion refractometry, and the mean phase-retardation through their mid-line was obtained from phase-change measurements on the bacilli mounted in dilute saline. Their mean thickness was calculated from these values.

With the second method, phase-change measurements were made on the bacilli mounted in protein mounting media as well as in saline, and the two sets of measurements were used to obtain values for their mean thickness.

Refractive index measurements

The method of immersion refractometry, first developed by Barer and Ross (1952), is very fully discussed by Barer and Joseph (1954 and 1955 *a*, *b*), and it need only be briefly described here. The bacteria were mounted in a series of solutions of Armour's bovine plasma albumin fraction V in distilled water, which had refractive indices ranging from 1·390 to 1·410. A Bellingham and Stanley pocket refractometer was used to measure the refractive index of each suspension at room temperature. This instrument has a built-in yellow filter with a transmission spectrum equivalent to the mean of the two sodium lines (589 mμ). The suspensions were made by mixing very small drops of the bacterial culture with the protein solutions, so that the latter were greatly in excess. There was never any measurable difference between the refractive indices of the suspensions and those of the protein solutions before the organism was added. Solutions of these concentrations had a tonicity approximately equal to that of a 0·25% NaCl solution (Ross, 1952).

These suspensions were examined with a phase-contrast microscope. In all the cultures and subcultures examined, the *L. bulgaricus* all appeared dark in solutions with refractive indices less than 1·400, and they all appeared bright in those with refractive indices greater than 1·409; which meant that the refractive indices of all the bacilli lay within these limits. In solutions with a refractive index of 1·404, approximately equal numbers of dark and bright bacilli were visible. This figure, therefore, was taken as the value of the mean refractive index of the living *L. bulgaricus*. It is equivalent to a cell-solid content of about 39% w/v.

The direction and wavelength of the light used for the phase-change measurements

Phase-change measurements were made with a Smith interference microscope of the bacilli mounted in 0·25% NaCl. In order to ensure that these measurements were the true diameters, the measurements were only made on bacilli that had their two ends in sharp focus simultaneously, and therefore lay nearly exactly at right angles to the optical axis of the microscope.

For critical phase-change measurements, it is necessary to use nearly monochromatic light of known wavelength. This was provided by an Ilford 807 (mercury green) filter used in conjunction with a tungsten 'pointolite' microscope lamp. The transmission of this filter with this light source was investigated with a spectroscope, because these gelatine filters often show slight individual variations from the maker's specifications. It was found to give a narrow green band with a maximum intensity at 542 mμ. This was therefore taken as the value for the mean wavelength of the light in which the observations were made.

Phase-change measurements by the extinction-point method

The most usual method of measuring phase-changes with the Smith interference microscope is by the extinction-point method. The analyser is first turned until the background (reference) field appears maximally dark; then it is rotated until the region of the object being measured appears maximally dark. The rotation of the analyser between the first and second position is directly proportional to the difference in phase between the light passing through the background (reference) field and the light passing through the region measured. This phase-change, in degrees, is equal to twice the angular rotation of the analyser.

This is quite a satisfactory method for making measurements on objects that show fairly large phase-changes in aqueous media, such as relatively thick tissue cells or highly refractile bacterial spores, because at the second position of the analyser the rotation has been big enough for the background field to appear quite bright. Against a bright field, the exact setting of the analyser at which the object being measured appears maximally dark can be judged by eye quite critically.

The method is less satisfactory, however, for objects showing phase-changes of less than about a fifth of a wavelength, such as most living bacterial vegetative cells mounted in water. It then becomes difficult for the eye to discern the exact setting at which the object appears maximally dark against a field that itself is not very bright. Under these conditions, there is a tendency for an observer to rotate the analyser too far, which makes the object appear in higher *contrast* to the background, but with less absolute depth of intensity. Therefore, without resorting to elaborate densitometric equipment, it is less easy to measure small phase-changes in this way.

For this reason, the extinction-point method of phase-change measurement, though fairly accurate, was hardly sufficiently so for measuring the living *L. bulgaricus* in order to obtain critical thickness estimations.

The Smith half-shade eyepiece

A recently developed accessory to the Smith interference microscope, the Smith half-shade eyepiece manufactured by Messrs. Charles Baker, enables phase-change measurements to be made much more easily and accurately than by the extinction-point method described above.

The optical principles of this ingenious device, which is used in the place of the ordinary monocular or binocular head of the Smith interference microscope, is illustrated in fig. 2, A. Light passing up the body tube of the microscope passes into the glass prism illustrated and impinges on the surface *aa'*. This is a glass/air surface which is set at such an angle to the incident light that it is all totally internally reflected. Not all the surface of the back of the prism *aa'* is, however, of plain glass. There is a specially treated region in the plane of the primary image formed by the field lens below the prism *e* in the form of a narrow horizontal strip on which metallic aluminium has been deposited. The light is internally reflected from this region also. The light i then all reflected from the surface *bb′* and passes out of the prism and throug a quarter-wave plate and a rotating polaroid analyser similar to those in th ordinary Smith interference microscope. The primary image, formed in th plane of the metallized strip, is then viewed by means of the additional incline accessory microscope illustrated.

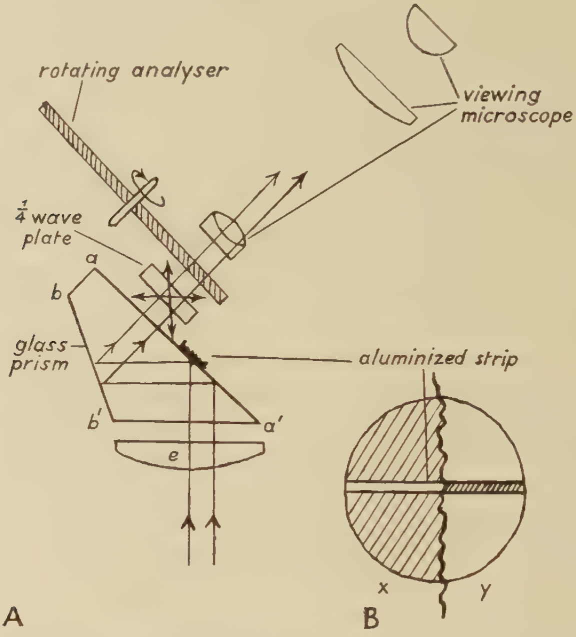

FIG. 2. A, the optical principles of the Smith half-shade eyepiece, manufactured by Messrs. Charles Baker, as an accessory to the Smith interference microscope. Redrawn from a sketch by Mr. F. H. Smith, and reproduced with his permission. For full explanation see text. B, the appearance of an empty field through the eyepiece in monochromatic light at two typical settings of the analyser: *x*, with the analyser set to give a dark background field and a bright strip image; *y*, with the analyser set so as to give a bright background field and a dark strip image

The working of the instrument depends on the fact that the light reflected from the metallized strip is (elliptically) polarized in a different manner fro the light that is reflected at the same angle from the unmetallized part of th surface *aa′* (Smith, 1954). The angles of the prism are arranged so that this has the effect of producing a final image in which there appears superimpose an image of the metallized strip that is permanently 120° out of phase with th rest of the field (fig. 2, B). This enables the image of the strip to be used as very sensitive intensity-matching device; and makes it possible to measur phase-changes very critically.

Because of the shift in phase between the two regions, with a white ligh source the image in the region of the half-shade strip will always appear of different interference colour from that of the rest of the field. In nearly monochromatic light, such as the mercury-green light used here, the relative intensities of the two regions will vary with the setting of the analyser (fig. 2, B).

The method of making phase-change measurements of the bacilli is illustrated in fig. 3, A, B, and in the photographs shown in fig. 4 (opposite p. 444).

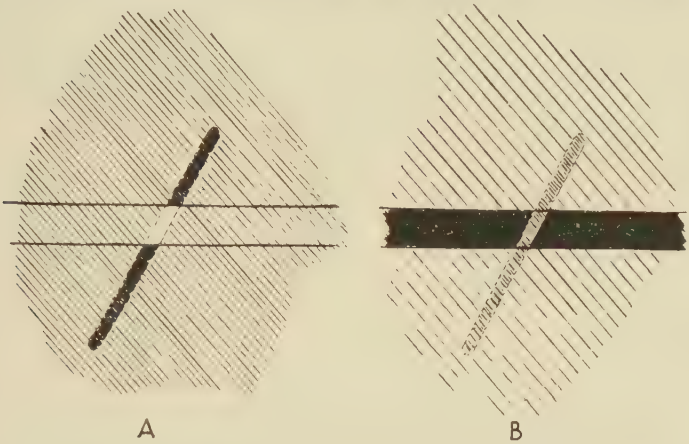

FIG. 3. Drawings illustrating the method of making a phase-change measurement on a living bacillus with the Smith half-shade eyepiece. A, the first setting of the analyser. The image of the background in the strip region matches the image of the rest of the background field in intensity. B, the second setting of the analyser. The image of the bacillus in the strip region matches the image of the bacillus in the rest of the field in intensity

The microscope stage is first adjusted so that the image of the bacillus being measured lies partly across the image of the strip. The analyser is then set so that the intensity of the background field in the strip region matches the rest of the background field (fig. 3, A; 4, A, C). At this setting the relative intensities of the image of the bacillus inside and outside the strip region are markedly different. The analyser is then rotated until the intensity of the image of the mid-line of the bacillus in the strip region appears to match that of the mid-line of the bacillus in the region outside the strip (figs. 3, B; 4, B, E). The rotation of the analyser between the first and second positions is proportional to the phase-change due to the maximum thickness of the bacillus; and the two positions can be judged with considerably greater precision than is usually possible with the extinction-point method. The phase-change in degrees is again equal to twice the angular rotation of the analyser.

Measurements

Phase-change measurements were made in this way of 10 different bacilli in each of the cultures that were examined; and the average of these 10 measurements was taken as the mean phase-change due to the *L. bulgaricus* in the culture. Bacilli from 14 different cultures mounted in 0·25% NaCl, mostly of different dates, were measured.

Two different kinds of 2-mm interference microscope objective were used, which are described by Smith and others elsewhere (1955). These were a 'shearing' objective and a 'double focus' objective. The 'shearing' objective was used for measuring the bacilli in preparations from four of the cultures. In theory, it is slightly superior to the 'double focus' objective because the separation of the light passing through the object and that passing through the background (reference) field is complete; but the advantage is almost negligible with objects so small as bacteria. In practice, there were no detectable differences between the measurements made with the two objectives, and the 'double focus' objective was used for most of the measurements on account of its superior resolution.

The distribution of the phase-change measurements made of the bacilli in this way in each of the 14 cultures examined were all very similar, and are shown in the Appendix, table 3. Added together, they showed a distribution very similar to that of a statistician's 'normal distribution', and this is illustrated in histogram form in fig. 5. It is certain that some of the variation in measurement was due to individual variations (in thickness or refractive index) between different bacilli. Part of it was also undoubtedly due to random errors in the phase-change measurements. In all the cultures, the values for the average phase-retardation of the population in each culture varied by a maximum of 4·8° (table 1).

The mean thickness in microns of the population in each culture t_1 was then calculated from the formula:

$$t_1 = \frac{\phi_1}{360} \times \frac{\lambda}{n - m_1}, \tag{3}$$

where ϕ_1 = the mean of the phase-retardation measurements on 10 bacilli expressed as an angle, n = the mean refractive index of the bacilli (1·404),

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FIG. 4 (plate). Photomicrographs taken with a Smith interference microscope and half-shade eyepiece, with a 2-mm 'double-focus' objective and an Ilford 807 (mercury green) filter of living bacteria from yoghourt cultures, mounted in 0·25% saline. b = *L. bulgaricus*; s = a strain of *Streptococcus*; d = an encapsulated *Diplococcus*.

A and B, a large number of bacteria near the edge of a drop, with one conspicuous bacillus of *L. bulgaricus* lying across the image of the half-shade strip. In A the analyser goniometer is set at 180°, and the image of the background field in the region of the half-shade strip matches the rest of the background (as in fig. 3, A). In B, the analyser goniometer is set at 150°. The image of the bacillus in the strip region appears nearly to match the part lying outside it, but actually it is rather darker. The rotation of the analyser from the position shown in A represents a phase-change of 60°. This means that this bacillus gives a phase-retardation of slightly less than this value.

C, D, and E, two bacilli of *L. bulgaricus*, a *Streptococcus* and an encapsulated *Diplococcus* lying on or near the image of the half-shade strip. The bacillus b is normal and healthy looking, but the bacillus b' is conspicuously thinner and is probably moribund. In C the goniometer is set at 180° giving a matching background (as in A). In D, the analyser goniometer is set at 150°, and it can be clearly seen that the region of the bacillus b within the strip is darker than that outside it. In E, the analyser is set at 153°, and the two regions of the bacillus b look more nearly matched; but the region within the strip is still actually darker. The rotation of the analyser from the position shown in C represents a phase-change of 54°. This means that the bacillus b gives a phase-retardation slightly less than this.

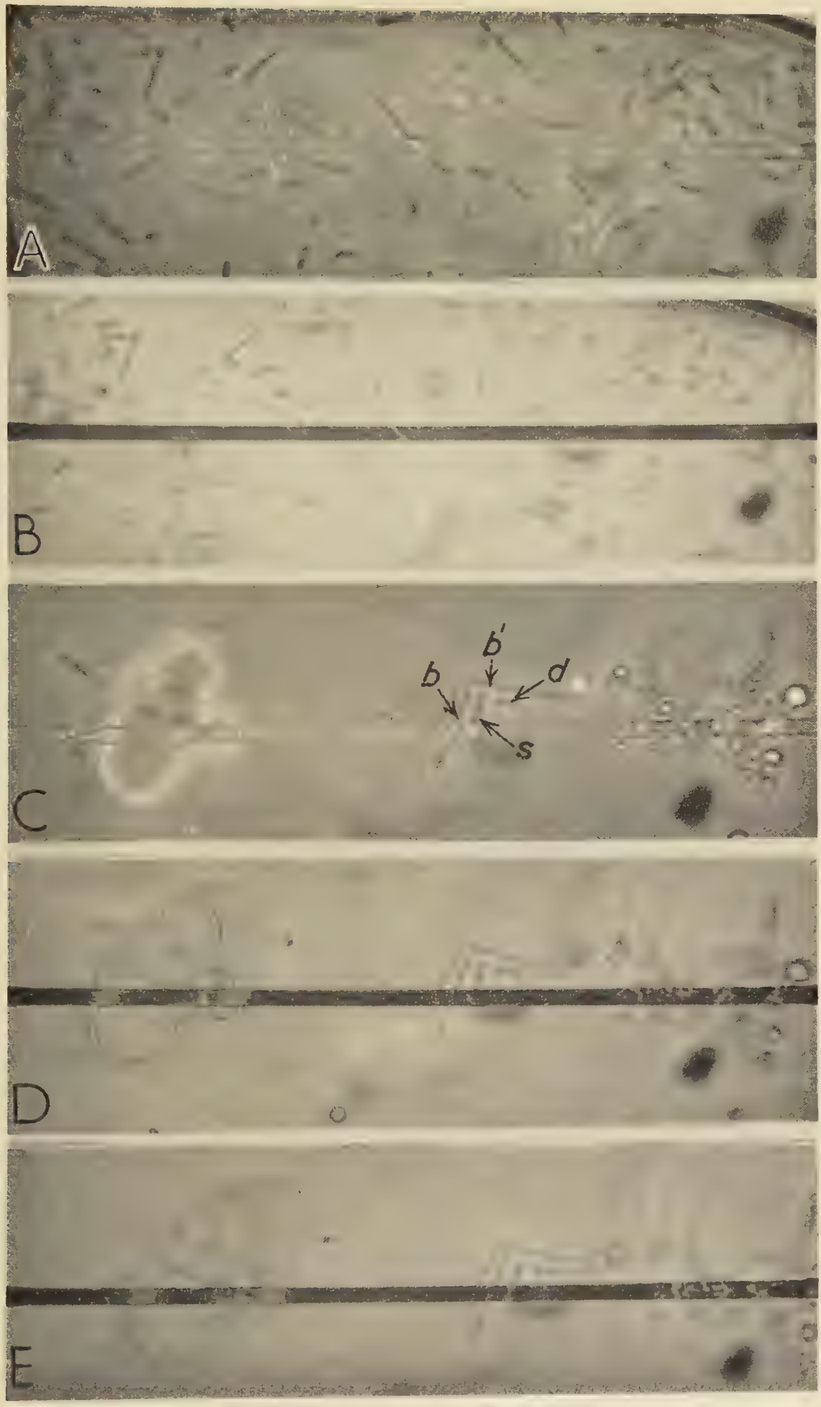

FIG. 4

K. F. A. ROSS

m_1 = the refractive index of the saline mounting medium (1·335), and λ = the mean wavelength of the light used (0·542 μ).

Table 1 shows the values for the mean retardation in each culture (ϕ_1) and the mean thickness (t_1) calculated from these. It will be seen that they vary by a maximum of 0·1 μ (i.e. from 1·13 μ in cultures 6 and 8 to 1·23 μ in cultures 2 and 12).

TABLE 1

The mean angular retardation of light, of wavelength 542 *m*μ, *through living* L. bulgaricus *from* 14 *different cultures mounted in* 0·25% *NaCl, and their mean thicknesses calculated therefrom.*

Culture no.	*Date of culture and subcultures*	*Date of examination*	*Mean retardation (ϕ_1) from 10 measurements on different bacilli*	*Mean thickness (t)*
Measurements with the 2-mm double-focus objective:				
			(*degrees*)	(μ)
1	11/7	20/7	55·8	1·21
2	11/7, sub. 16/7	21/7	56·4	1·23
3	11/7, sub. 16/7 and 24/7	27/7	52·6	1·14
4	25/7	28/7	52·8	1·14
5	25/7	29/7	53·2	1·15
6	25/7, sub. 27/7	29/7	52·2	1·13
7	25/7, sub. 27/7	29/7	53·6	1·16
8	25/7, sub. 27/7	29/7	52·0	1·13
9	25/7, sub. 27/7	30/7	52·4	1·14
10	25/7, sub. 27/7	30/7	53·6	1·16
Measurements with the 2-mm shearing objective:				
11	11/7, sub. 16/7	21/7	54·6	1·19
12	11/7, sub. 16/7 and 24/7	27/7	56·8	1·23
13	25/7	28/7	52·6	1·14
14	25/7	29/7	54·2	1·18

Possible sources of error

Refractive indices can be measured very critically by the method of immersion refractometry already described, and those of the bacilli were certainly accurate to the nearest 0·001. Consequently, the accuracy of the values for the mean thickness was not limited by these measurements but by the accuracy of the phase-change measurements.

The random error in matching the bacillus and field with the half-shade eyepiece was small. The first setting of the analyser, the matching background (fig. 3, A), could be judged to the nearest 1° on the goniometer scale on the analyser. Repeated measurements were made on several individual bacilli to determine the maximum range over which the two regions of each bacillus appeared to match each other at the second position of the analyser (fig. 3, B). The extreme range was found to be 4°, and it was more usually 3°, on the goniometer scale. This meant that the maximum error for both settings was $\pm 2\frac{1}{2}°$ on the goniometer scale, which represented a maximum error in phase-change measurement of $\pm 5°$, or about one-seventieth of a wavelength. By comparing the photographs D and E in fig. 4 (which are printed in about the same degree of contrast as can be appreciated by the eye looking down the microscope), it can be seen that a difference of 3° on the goniometer scale can usually be appreciated fairly easily.

As well as these random errors, there were several reasons to suggest that small systemic errors in phase-change measurement might have been made.

The first of these arose from the fact that, rather unexpectedly, there was no very noticeable intensity gradient between the mid-line and edges of any of the bacilli when they were being examined and measured with the half-shade eyepiece. From their circular cross-section one might have expected the intensity across their width to have varied in a manner similar to that in the curve shown in fig. 1, B. In fact, there was a nearly uniform intensity across the whole visible width of each bacillus, and thus the intensity curve resembled more closely that shown in fig. 1, A. This can also be seen in all the photomicrographs in fig. 4. This appearance seemed to be confirmed by a microdensitometer trace on the photomicrograph negatives that was kindly undertaken by Dr. P. B. M. Walker in the M.R.C. Biophysics Unit at King's College, London; but the slit of the densitometer was barely narrow enough for this to be absolutely conclusive.

There are three possible explanations for this apparent lack of gradient or flat-topped curve.

(1) The bacilli may have been too small for the phase-change gradient across them to have resolved as an intensity gradient with the objectives used. In this case, the uniform intensity over the whole image would represent the integrated phase-change across the whole object, and the intensity at the mid-line would not represent the phase-change due to the true diameter. A correction factor would therefore be needed in deducing the latter.

(2) There may have been a negative density gradient within the bacilli from surface to centre (Mitchison, 1956). If the refractive index of the outer regions of a bacillus was relatively high and fell off towards the centre, one might expect to find relatively little difference between the phase-changes at the thinner, more refractile edges and that at the thicker but less refractile centre, even if the phase-change gradients were resolvable.

(3) It could be due to the fact (pointed out by Mitchison, Passano, and Smith, 1956) that the relationship between the phase-change caused by an object and the intensity of its image under the interference microscope in monochromatic light is only a linear one if the phase-changes are less than 45°. From table 1 it can be seen that the phase-changes measured through the middle of the bacilli are greater than this. It is therefore quite possible that, at certain settings of the analyser, phase-changes through the edges and mid-line of a bacillus, though different, could give rise to very similar depths of intensity.

If either of the first two explanations are true, one would expect there to be no noticeable intensity gradient visible at *all* settings of the analyser: but this is not what is actually found. At certain settings, notably at the position of 'minimum visibility' (i.e. when the centre of a bacillus matches the background intensity) a distinct intensity gradient between the edges and centre can be seen. This suggests that the third explanation is probably the correct one.

When these results were first reported in a preliminary publication (Ross, 1955) it was assumed that the first explanation was correct, and that the phase-change gradient across the bacilli was unresolved. An integrated phase-change correction factor was therefore applied, and all the phase-change measurements were multiplied by $2/\sqrt{\pi}$ ($= X1{\cdot}13$). It now seems that this was not necessary, and the values shown in table 1 are not corrected in this way. However, table 4 in the Appendix shows the values for the mean thickness of the bacilli in each culture with this correction applied. It will be seen that they are all about 0·15 μ higher than those shown in table 1.

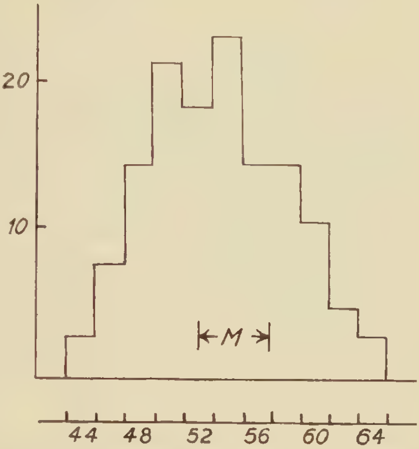

FIG. 5. A histogram showing the distribution of 140 phase-retardation measurements made on individual bacilli of *L. bulgaricus* from 14 separate cultures mounted in 0·25% NaCl. Ordinate = number of bacilli. Abscissa = phase retardation. *M* = the maximum range of the values for the mean phase-retardation from 10 measurements in each culture

A second cause of systematic error can be more simply explained, but is more important because it is more probable. This was due to the difficulty of judging by eye the second position of the analyser where the two regions of the bacillus, within and outside the half-shade strip, exactly matched each other. In fig. 3, B the drawing of the bacillus has been uniformly shaded throughout its length, but the region within the half-shade strip appears slightly brighter than the rest of it because of the adjacent dark regions of the strip. Conversely, although in fig. 4, B, E, the bacillus *appears* of about uniform intensity throughout its length, if one blocks out the image of the strip and background field by pieces of paper placed on each side of the image of the bacillus, it will be clear that this is not true, but the part of the bacillus in the region of the half-shade strip is actually darker than the rest. This means that, instead of achieving a true match, the goniometer has been turned too far. Therefore, when judging by eye, an observer tends to *over*-estimate slightly all the phase-change measurements by an amount that is difficult to quantify.

It would obviously be possible to arrive at the true values of the phase-change by photography, with photomicrographs taken at every few degrees setting of the analyser; but in practice this would be extremely laborious because the living bacilli in the suspensions are not often stationary for long enough for a sharp photographic exposure of several seconds' duration. It was therefore desirable to use some other method, the accuracy of which could be checked, because in this way the systematic error could be determined.

Thickness from phase-change measurements in two different mounting media

The second method of measuring the mean thickness of the *L. bulgaricus* had the advantage just mentioned, that its accuracy could be checked by an independent method.

Bacilli from 10 of the 14 cultures measured by the previous method were also mounted in solutions of Armour's bovine plasma albumin fraction V in distilled water, that had refractive indices varying from 1·365 to 1·376. Phase-change measurements were made (with the 2-mm 'double focus' objective) of 10 bacilli from each culture in these mounting media, and these were compared with the measurements made in saline. Values for the mean thickness of the bacilli in each culture t_2 were then calculated from the formula (Barer, 1953)

$$t_2 = \frac{\phi_1 - \phi_2}{(m_2 - m_1)360} \times \lambda, \tag{4}$$

where ϕ_1 = the mean of the phase-retardation measurements of 10 bacilli mounted in saline, ϕ_2 = the mean of the phase-retardation measurements on 10 bacilli mounted in the protein mounting medium, m_1 = the refractive index of the saline mounting medium (1·335), m_2 = the refractive index of the protein mounting medium, and λ = the mean wavelength of the light used (0·542 μ).

Table 2 shows the values for the mean of the phase-change measurements made of the bacilli from each culture in the saline (ϕ_1) and the protein mounting media (ϕ_2), the refractive indices of the protein solutions used (m_2), and the figures for mean thickness calculated from these (t_2).

It will be seen that all the figures for the mean thickness are a little lower than those obtained by the former method. It is difficult to be certain of the reason for this, but the most likely explanation is that the systematic errors in the phase-change measurements due to the difficulties of matching were slightly different in the case of the bacilli mounted in protein.

From table 2 it can also be seen that, with the exception of culture no. 6, the figures for the mean thickness of the bacilli in each culture differ by a maximum of 0·12 μ, ranging from 1·02 μ in cultures 9 and 10 to 1·14 μ in culture 7. The figure for culture 6 (0·89 μ) is so much outside the range of the rest that it seems probable that the protein solution in which the second phase-change measurements were made was accidentally contaminated with water and diluted after its refractive index had been measured. There is, however, no certain evidence that this is what happened; and the figures in question are therefore included in brackets in the table for the reader to evaluate them as he thinks fit.

As in table 1, no correction was applied to the phase-change measurements for integration, but with this method such a correction would not make a great deal of difference to the results. Table 4 in the appendix shows the figures for mean thickness with this correction applied, but, for reasons already discussed it is very unlikely that this correction was necessary.

TABLE 2

The mean angular retardations of light, wavelength 542 mμ, through living L. bulgaricus *from 10 different cultures and sub-cultures mounted in 0·25% NaCl and in protein solutions of various refractive indices; and the values for their mean thickness and mean refractive index calculated from these measurements*

Culture no.	*Mean retardation in saline* (ϕ_1)	*Mean retardation in protein* (ϕ_2)	*Refractive index of protein solution* (m_2)	*Mean thickness* (t_2)	*Mean refractive index of bacilli* (n) *calculated from* t_2, ϕ_1 *and* m_1
	(*degrees*)	(*degrees*)		(μ)	
1	55·8	32·8	1·365	1·12	1·410
2	56·4	25·8	1·376	1·13	1·410
3	52·6	23·4	1·374	1·11	1·407
4	52·8	24·8	1·375	1·10	1·407
5	53·2	25·0	1·373	1·11	1·403
6	52·2	(28·0)	(1·376)	(0·89)	(1·423)
7	53·6	24·4	1·373	1·14	1·406
8	52·0	23·4	1·376	1·03	1·411
9	52·4	28·4	1·370	1·02	1·413
10	53·6	31·0	1·368	1·02	1·414

Mean refractive index of *L. bulgaricus* from immersion refractometry: 1·404

This 'double immersion' method was similar to that employed by Barer in 1953 for measuring the thickness of epithelial cells from the human mouth, except that, instead of the phase-change measurements being made of the same individual cells suspended successively in the two mounting media, they were made on a different sample of the bacterial culture in each medium. This was done because it was not possible to keep the same bacteria in the field while changing the mounting medium.

CONCLUSIONS AND DISCUSSION

The accuracy of the mean thickness figures obtained

As has already been mentioned, the 'double immersion' method just described has the great advantage that its accuracy can be checked by an independent method. This is because it enables one to obtain a second figure for the mean refractive index of the bacilli in each culture which is independent of that obtained experimentally by immersion refractometry (1·404). These refractive indices can therefore be used as a check on the validity of the thickness measurements.

The mean refractive index of the bacilli in each culture, n, can be calculated from the formula

$$n = \frac{\phi_1}{360} \times \frac{\lambda}{t_2} + m_1, \qquad (5)$$

where the symbols are the same as in formulae (3) and (4). Equally well, it could be calculated from the same formula (5), using ϕ_2 and m_2.

The final column of table 2 shows the mean refractive indices of each culture calculated in this way. Except in the case of culture 6, it can be seen that all these figures (ranging from 1·403 in culture 5 to 1·413 in culture 10) are very close to the value of 1·404 obtained on some of the cultures by immersion refractometry. The greatest departure (apart from culture 6) is shown in the case of culture 10, where the figure for refractive index differs from that obtained by immersion refractometry by 0·009. Even this, however, is only equivalent to a difference of about 5% w/v in the solid content of the bacterium; and it represents an error in thickness measurement of 0·14 μ if reckoned on the basis of the phase-change measurements in saline, and 0·11 μ if reckoned on the basis of the phase-change measurements in the protein.

Since (apart from culture 6) this is the maximum refractive index difference, one may conclude that the figures for the mean thickness obtained by the double immersion method are not very often wrong by more than 0·1 μ. Rarely they may be wrong by a maximum of 0·15 μ. Those obtained by the first method are on this reckoning probably about 0·1 μ too high.

It therefore seems justified to conclude that both methods enable one to make a more accurate estimate of the mean thickness of living bacteria than is possible by direct measurement with an eyepiece micrometer, provided that enough measurements are made (10 are rather few) and that the considerations discussed above are borne in mind.

The effect of tonicity

As has already been mentioned, an attempt was made to allow for the effective tonicity of the protein solutions, in which half the measurements were made, by using a 0·25% saline solution instead of distilled water as the mounting media for the other measurements. This was probably unnecessary, since bacteria are not usually very sensitive to small changes in tonicity. To check this, bacilli from one of the cultures (no. 7) were measured when mounted in distilled water. The range of phase-change measurements was very similar to those made of the bacilli in saline, and their mean was 52·6° as compared with 53·6° for the bacilli mounted in saline (table 1). One may therefore conclude that the adjustment for tonicity, which was only approximate, was hardly necessary at all.

The detection of dead and moribund bacteria

All the cultures and subcultures measured were freshly grown, and were at a logarithmic stage of growth; so that there were very few dead bacteria present. However, several old cultures, that had been left on the bench at room temperature for a week or more, were also measured. These were found to contain a considerable number of bacilli that gave an appreciably higher phase-change than normal; and these were probably dead or moribund cells that had begun to shrink in width. Indeed, many of the bacilli in these cultures were visibly shrunken and looked dead. It is therefore possible that, under certain circumstances, these techniques might be useful for checking the 'health' of a bacterial culture, since this may be a particularly sensitive method of detecting the presence of moribund cells.

Other bacterial width-measurements made by interferometry

Ross and Billing (1957) used the 'double immersion' method described above, and obtained values for the mean thickness of the living spores of three different spore-forming bacilli. In two successive experiments (each involving 10 measurements in each medium) performed with each species, the values for the mean thickness of *Bacillus cereus* were 1·08 μ and 1·10 μ; for *B. cereus var. mycoides* 0·96 μ and 1·02 μ, and for *B. megaterium* 1·00 μ and 0·95 μ. In the two latter organisms the spore size varied considerably, but the values for *B. cereus* are probably very near to the true thickness of the majority of the individual spores.

Previous estimations of the size of bacteria

Previous estimations of the size of bacteria have almost all been based on eyepiece-micrometer measurements or on the measurement of photomicrographs; but the difficulties of direct measurement, discussed in the introduction, do not seem to have been fully appreciated. Figures are given for the widths of living bacteria (e.g. by Knaysi, 1945), often to a wholly unjustifiable number of decimal places, without any mention of the particular points on the diffraction gradients from which the measurements were taken.

An exception to this, however, is the interesting work of Dubin and Sharp (1944), who estimated and compared the widths of a number of air-dried *B. megaterium* in visible light and in electron-micrographs. For the measurements in visible light, they estimated the width of a bacillus from the middle of the main diffraction gradients at its edges; but they also expressed some doubt as to the justifiability of doing this. Measurements based on this criterion agreed very closely with the widths of the main dense regions of the same bacilli in the electron-micrographs.

At first sight, this would seem to invalidate the contention, put forward in the introduction, that width measurements of cylindrical bacilli made from the middle points of the main diffraction gradients will give an under-estimation of their true width. This, however, is not so, because the electron-micrographs also showed, outside the main dense part of each bacillus, a paler zone of appreciable thickness, with a width of about 5–10% of that of the main dense part. This, for reasons discussed by Ross and Deutsch (1957), is probably a surface deposit, but whether this is so or not, it will certainly contribute to the diffraction pattern seen in visible light and modify it by making it wider. The width of the bacilli indicated by the diffraction gradient would therefore include at least part of the width of this pale zone.

Therefore, these observations seem rather to confirm the contention that bacteria measured by eye in visible light tend to look slightly smaller than the really are.

Richards (1948) claims that bacteria under the phase-contrast microscop can be measured to an accuracy of ±0·05 μ because phase-contrast image nearly always have sharper edges than those of an ordinary microscope; bu this is almost certainly erroneous. The sharp edges of these images are optica artifacts due to the incomplete separation of the direct and diffracted light in the phase-contrast system; and they could in fact be regarded as modified (and greatly steepened) diffraction gradients of the kind already described (Barer and Joseph, 1955*b*). They therefore do not usually represent the actual boundaries of a microscopic object, but, in the case of bacteria of circular cross-section, they probably lie some distance inside these boundaries.

I should like particularly to thank Dr. J. R. Baker of the Department of Zoology and Comparative Anatomy, Oxford, Dr. J. M. Mitchison of the Department of Zoology and Natural History, Edinburgh, and Mr. F. H Smith of Messrs. Charles Baker for the active interest they have taken in the course of this investigation, and for their encouragement and numerous helpful suggestions.

The Smith interference microscope used was provided out of a grant to the author from the London University Central Research Fund, and so was the Smith half-shade eyepiece used to obtain the photographs shown in fig. 4. The Smith half-shade eyepiece actually used for the measurements was kindly loaned to the author at an earlier date by Messrs. Charles Baker.

I would also like to thank Dr. P. M. B. Walker of the M.R.C. Biophysics Unit at King's College for undertaking the microdensitometric measurements mentioned on page 446, Miss Eithne Whittaker for help in the earlier stages of the investigation, Mrs. Barbara Holland for typing my manuscript, and Dr. T. H. K. Barron of the Division of Pure Physics, National Research Council, Ottawa, for a valuable piece of advice.

Finally, I would like to thank Dr. A. T. B. Mattick of the National Institute for Research in Dairying, Shinfield, for identifying the organism provided by the Laboratoires 'Yalacta' in Paris stated to be *Bacillus bulgaris*, as a typical strain of *Lactobacillus bulgaricus.*

APPENDIX

TABLE 3

The measured angular retardations of light, of wavelength 542 *mμ, through ndividual bacilli of* L. bulgaricus *in* 14 *different cultures and subcultures, ounted in* 0·25% *saline, and the mean angular retardation in each of these*

Culture no.	*Measured φ*										*Mean φ*
	(degrees)										*(degrees)*
Measurements with 2-mm double-focus objectives:											
1	48	48	50	52	56	58	60	60	62	64	55·8
2	46	50	54	54	56	58	60	60	62	64	56·4
3	46	46	50	50	52	54	56	56	58	58	52·6
4	48	50	50	50	52	52	54	56	56	60	52·8
5	46	50	50	52	52	54	54	58	58	58	53·2
6	46	50	50	50	52	52	54	56	56	56	52·2
7	48	50	52	52	52	54	56	56	58	58	53·6
8	46	48	48	50	50	50	50	54	56	58	52·0
9	48	48	48	50	50	52	54	56	58	60	52·4
10	44	46	48	50	50	52	54	54	58	60	53·6
Measurements with 2-mm shearing objective:											
11	44	46	52	54	54	56	56	60	62	62	54·6
12	52	52	54	54	54	58	58	60	62	64	56·8
13	44	48	50	52	54	54	54	54	58	58	52·6
14	48	48	48	52	52	54	54	54	56	60	54·2

TABLE 4

Values for the mean thickness of living L. bulgaricus *from a number of different ultures, measured by the 'single immersion' and 'double immersion' methods as tables* 1 *and* 2, *but with an integration correction of* $X\ 2/\sqrt{\pi}$ (= X1·13) *applied to all the phase-change measurements*

Culture no.	*Mean thickness (t_1') calculated from single sets of phase-change measurements in* 0·25% *NaCl, and a mean r.i. of* 1·404 *measured by immersion refractometry*	*Mean thickness (t_2') calculated from two sets of phase-change measurements in* 0·25% *NaCl and in protein solutions of various refractive indices*
	(μ)	(μ)
1	1·37	1·33
2	1·39	1·19
3	1·29	1·28
4	1·29	1·17
5	1·30	1·23
6	1·28	(1·01)
7	1·31	1·29
8	1·28	1·19
9	1·29	1·17
10	1·31	1·17
11	1·34	. .
12	1·39	. .
13	1·29	. .
14	1·33	. .

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The Cuvierian Tubules of *Holothuria leucospilota*

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SUMMARY

Each Cuvierian tubule of *Holothuria leucospilota* consists of a bounding layer of large cells beneath which are bundles of fibres embedded in a clear ground-mass. Present also is an axial cavity containing a core of morula-shaped cells. Immediately below the outer layer longitudinal muscles occur and, internal to these, muscle-bands which run spirally are found.

The cells of the outer layer contain either granulated compartments or angular bodies. Both compartments and angular bodies contain proteinaceous materials. When a tubule is stretched in sea water the cells of the outer layer split and their contents form an amorphous mass which has strong adhesive properties.

The fibres present in the connective tissue of the tubules are collagenous and are associated with acid polysaccharides part, at least, of which are mucopolysaccharides. A polysaccharide which can be removed by testicular hyaluronidase is present in the interfibrillar ground-mass. The chemical constitution of the tubule connective tissue is similar in many respects to that of vertebrate connective tissue.

The mode of discharge and elongation of the tubules is discussed.

INTRODUCTION

SOME species of holothurians possess tubules which, when ejected into sea water, acquire great adhesive powers rendering them capable of entangling many predatory animals. These tubules, named after their discoverer, Cuvier, have for long aroused the attention of zoologists. Particular attention has been paid to their structure by Hamann (1883), Jourdan (1883), Hérouard (1889), Cuénot (1891), and Ludwig and Barthels (1892), but the views of these authors are often conflicting. Likewise, there has not been general agreement on the mechanism or mechanisms bringing about discharge and elongation of the tubules (Jourdan, 1883; Hérouard, 1889; Cuénot, 1891; Minchin, 1892, and Mines, 1912).

Observations on the structure and mode of discharge of the Cuvierian tubules of *Holothuria leucospilota* are included in this paper, but the prime objective has been a study of the structure, chemical constitution, and role of the connective tissue which is especially prominent in the tubules of this species.

MATERIAL AND METHODS

H. leucospilota Brandt is one of the commonest and most conspicuous animals found on the reef flat at Heron Island in the Capricorn Group, Queensland. Sometimes specimens are encountered which are lying fully exposed on the coral sand between coral clumps and can be collected with ease. More frequently, the major portions of their sausage-like bodies are lodged beneath ledges of coral and only the anterior 4–6 in. with its crown of tentacles is exposed. If such animals are grasped firmly, a quick tug usually dislodges

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them and they can be manœuvred into large enamel dishes. Careful transport of captured animals to the laboratory is essential, as rough handling or sudden jarring may cause them to eject their Cuvierian tubules. The holothurians may be kept for several days in well-aerated aquaria.

Specimens from Heron Island were used initially in the present investigations. Subsequently, they were secured from Caloundra (southern Queensland), where they occur in rock pools near low-water mark.

Methods used in particular investigations are described under relevan headings.

MODE OF EMPLOYMENT AND GROSS STRUCTURE OF THE CUVIERIAN TUBULES

When grasped, a specimen of *H. leucospilota* usually loops its body, point its anal end in the direction of the source of irritation, and contracts forcibly the body-wall musculature. Sea water is expelled via the anus and this is then followed by the sudden appearance of a mass of white threads, which, on contact with sea water, resolve themselves into a number of white tubules These swell, writhe, and elongate rapidly in the general direction of tidal flow and some attain almost a metre in length before breaking away from the holothurian. The ejected tubules are intensely sticky and are generally considere to be organs of defence since they are capable of entangling predatory animal such as crabs and fish.

Usually the tubules are exuded in short bursts but eventually, if irritation is sufficiently strong, the straw-coloured or reddish gonad protrudes through the anus and shortly afterwards most of the viscera are voided.

Specimens were placed carefully in a dish containing sea water to which magnesium sulphate crystals had been added. After 3–4 h the animals wer extended, flaccid, and inert, and were considered suitably anaesthetized They were removed to a dissecting dish and a median longitudinal incision made through the body-wall thereby revealing the internal organs.

Intertwined whitish but translucent tubules were noted aborally. Thes were each free at one end which lay in the body-cavity. The other end of eac was linked by a thread of tissue to the common stem of the respiratory tree or to the base of the left respiratory tree (fig. 1). The exposed tubules did nc adhere to objects placed in contact with them and they were not very stick to the touch.

The tubules averaged 13 cm in length. The narrower ones each possesse a very fine cross-striation and a darker central core which ran the length of th tubule. In the thicker tubules the cross-striated appearance was difficult t detect because the darker central core appeared to be folded on itself. Th number of tubules present in each of fifteen specimens taken at randon ranged from 110 to 147.

Some specimens were allowed to eject bursts of tubules and were the anaesthetized with magnesium sulphate. Subsequent internal examinatio revealed the presence of a variable number of tubules still attached to th respiratory trees and the stems from which others had broken away. Thos tubules nearer the cloaca were always the first to be ejected. Although some of the specimens had ejected up to eight bursts of tubules all were found to possess some tubules which were still attached. In all cases the cloacal wall was torn between its junctions with the rectum and with the common stem of the respiratory trees.

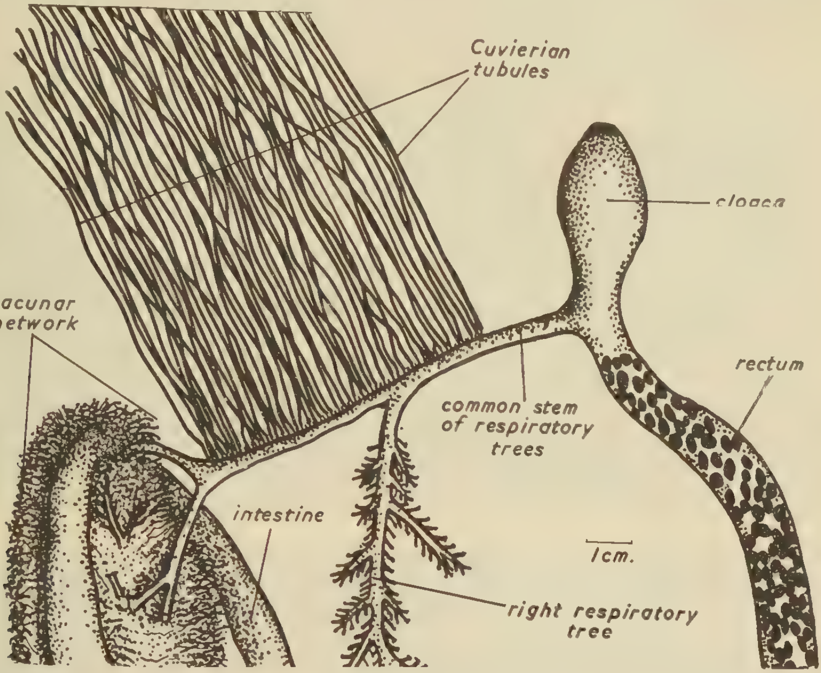

FIG. 1. Portion of the alimentary canal and associated structures of *H. leucospilota* showing the region of attachment of the Cuvierian tubules

When ejected into dilute NaOH (pH = 8·2) distilled water or fresh water the tubules were not particularly sticky to the touch nor did they elongate to any extent. They did, however, exhibit a marked tendency to clump together.

If an animal was lifted from sea water the tubules which it ejected on to a dry hand were not very sticky and they did not swell. Yet, when these same tubules were detached from the holothurian and lowered into sea water, they swelled, separated from one another, elongated, and became intensely sticky.

If tubules which had been ejected into distilled water were rubbed between the fingers a sticky outer layer was removed and a strong fibrous core remained.

MICROSCOPIC STRUCTURE OF THE TUBULES

Tubules, taken from the body-cavity and mounted in a little coelomic fluid, were examined microscopically. The edge of each tubule had an undulating appearance and the surface of each was ridged and furrowed (fig. 2, A). The ridges and furrows ran spirally around each tubule. Also the tubules varied in diameter, some being as narrow as 0·25 mm, but the diameters of the majority ranged from 0·7 to 2 mm.

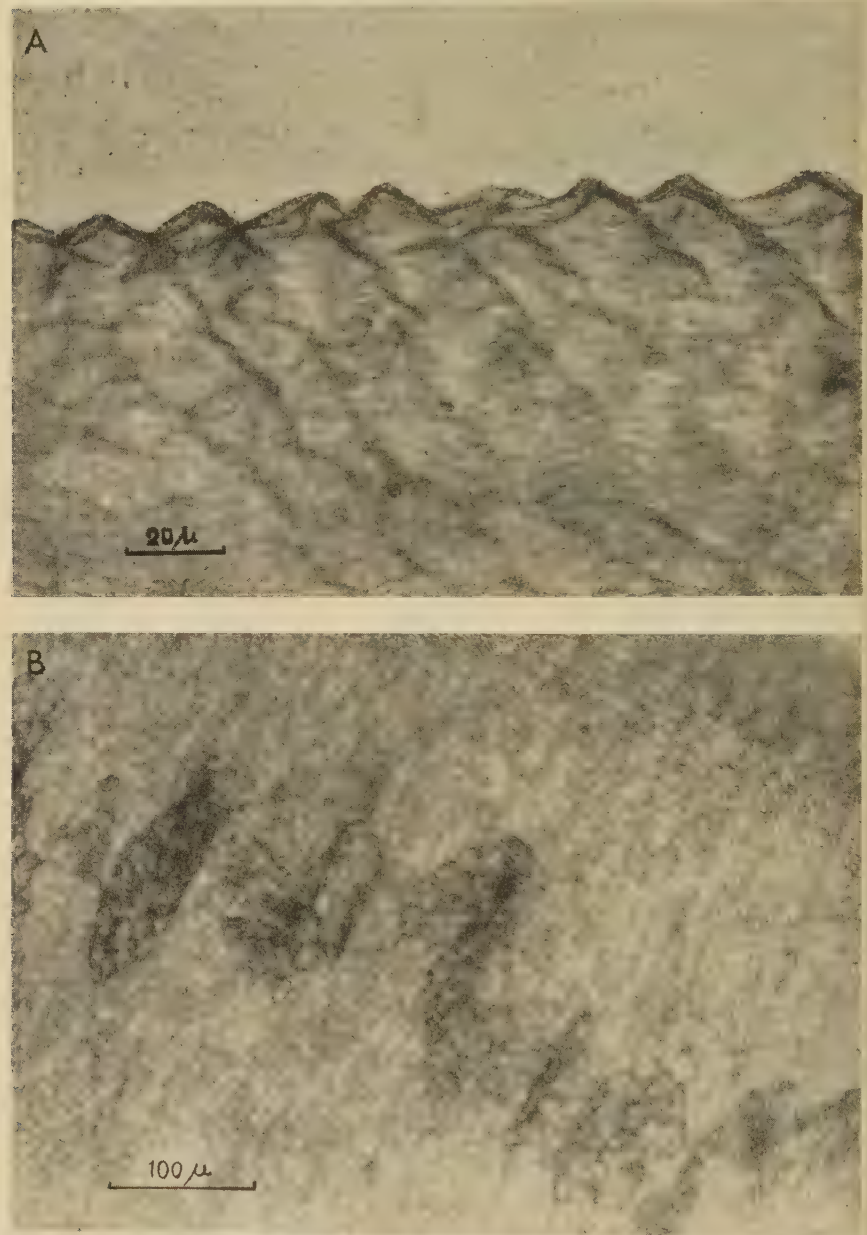

FIG. 2. A, photomicrograph of the edge and portion of the surface of a fresh Cuvierian tubule of *H. leucospilota* showing alternating ridges and furrows. B, photomicrograph of the central region of a whole mount of a thick Cuvierian tubule of *H. leucospilota* showing the spiral central core of morula-shaped cells

In each tubule a central core was evident. This consisted of packed cells which varied in size but averaged 27 μ long by about 9 μ wide. Although most were elongated in this fashion, some were spherical and had diameters of about 15 μ. Three to five such cells were packed side by side across the central core which had an average diameter of about 45 μ in slightly stretched tubules. However, the width of the core varied with the diameter of the tubules. In the thicker unstretched tubules it had a diameter of about 70 μ and followed a spiral course (fig. 2, B). When such tubules were stretched, the core straightened and narrowed, and it was then observed that the cells were independent units which moved apart from one another as the tubule was stretched.

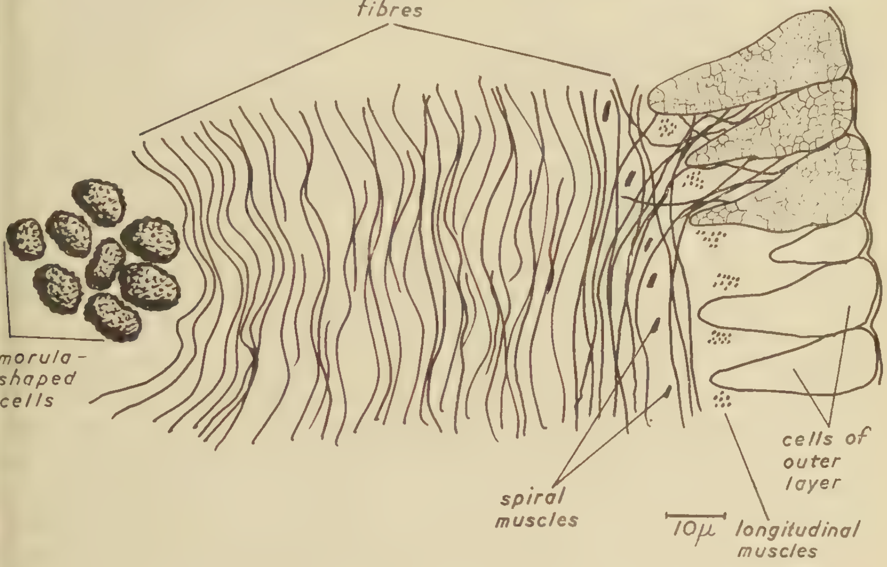

FIG. 3. Part of a transverse section through a Cuvierian tubule of *H. leucospilota*

The tubules were torn with dissecting needles and this resulted in the emergence of morula-shaped cells from the central core. These cells possessed a number of refringent globules and were similar to cells seen in cursory examination of the coelomic fluid. Cells of the same type were also found outside the central core nearer the periphery of the tubules. Many of these cells were brownish and misshapen, and frequently their globules had been released and were disposed in straight lines.

Transverse and longitudinal sections of tubules fixed variously with Bouin's fluid, Heidenhain's 'Susa' fluid, Carnoy's fluid, and 5% formalin in sea water were prepared and stained with a variety of stains. Mallory's aniline blue collagen stain and Heidenhain's 'azan' modification produced good results.

Sections (fig. 3) revealed that each tubule consisted of a bounding layer of large cells beneath which were bundles of fibres embedded in a clear ground-mass. Present also was an internal cavity containing a core of morula-shaped cells which usually presented a confused picture owing to cytolysis caused by the fixatives used. Immediately below the outer cell-layer, longitudinal muscles were observed and, just internal to these, muscle-bands which ran spirally were noted. The muscles passed through the fibre matrix.

THE OUTER LAYER OF CELLS

(*a*) *Histology*

On the outside of each tubule was a very thin membrane which was often difficult to distinguish because it was frequently disrupted and because it stained similarly to the boundaries of the underlying cells. In sections stained with Mann's methyl-blue eosin stain, it appeared bluish under the ordinary microscope, but examination of these sections with the phase-contrast microscope revealed it as a greenish layer on the outside of the underlying cells whose boundaries remained bluish. Aggregations of granulated material were observed in the membrane under oil immersion, but there was no evidence of the presence of nuclei. Such a nucleated membrane covers the tubules of *Actinopyga agassizi* (Selenka) described recently by Hyman (1955).

Hamann (1883) and Jourdan (1883) maintained that a coelomic epithelium covered an underlying layer of glandular cells in the tubules of holothurians. However, Cuénot (1891) noted that all nuclei present in this 'epithelium' in the case of *H. impatiens* Forskal were nuclei of coelomocytes which attached themselves to it. Ludwig and Barthels (1892), after studying several species of holothurians, reached the conclusion that the covering of the tubules is not a separate cell layer but is part of the underlying cells. The present observation is in accord with their finding.

The cells underlying the membrane in *H. leucospilota* occurred in a single layer and averaged 18 μ in length measured along the long axis of the tubule. They were about 10 μ wide, but tapered towards the core of the tubule, and their height ranged from 15 to 35 μ. The cells abutted one another below the surface membrane but internally they were separated by fibrillar material (fig. 3). Their general appearance in cross-sections of tubules is shown in fig. 4 and in longitudinal sections in fig. 5.

In slightly stretched tubules the cells were disposed in parallel rows which ran the length of each tubule and which described the path of a very loose spiral. At the same time the cells were arranged in parallel rows which followed the path of a tight spiral (fig. 6).

Compartments containing numerous granules were found in the cells or, alternatively, the cells were crowded with angular bodies of much the same dimensions. Occasionally compartments with granules and angular bodies coexisted in the same cell. In the thicker tubules the cells of the outer layer contained angular bodies only. It seems probable that the compartments with granules precede and give rise to the angular bodies.

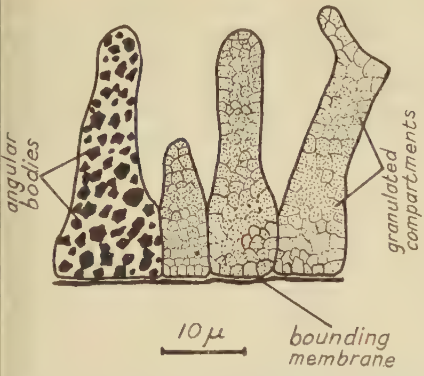

FIG. 4. Four cells of the outer layer seen in transverse section

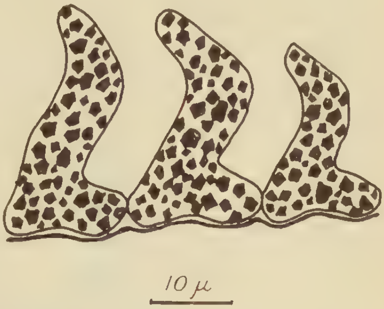

FIG. 5. Three cells of the outer layer seen in longitudinal section

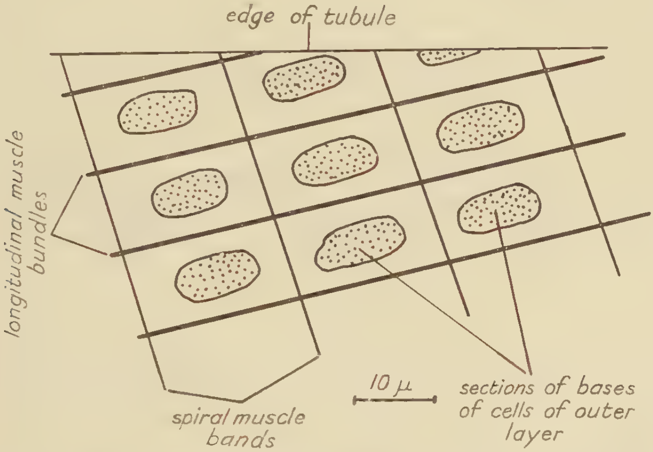
edge of tubule
longitudinal muscle bundles
spiral muscle bands
10 μ
sections of bases of cells of outer layer

FIG. 6. Diagram showing the arrangement of the cells of the outer layer with respect to the longitudinal and spiral muscles

Also in sections of the thicker tubules which had been stretched before fixation the cells of the outer layer seemed to have split along their long axes conferring upon them the appearance shown either in fig. 7 or fig. 8.

Jourdan (1883), Hérouard (1889), and Cuénot (1891) believed that each cell of the outer layer was tubular and bent upon itself so that the walls of each transverse surface ridge consisted of the halves of adjacent cells. Such tubular cells were not observed in *H. leucospilota*, and it is possible that these authors may have observed split cells.

In sections of tubules which had been ejected into sea water before fixation it was noted that most of the cells of the outer layer had split and their contents (granulated compartments and angular bodies) had been converted into an amorphous granulated mass. This is the material which confers upon the tubules their great powers of adhesion.

Nuclei were observed in many of the cells of the outer layer, but the position of these nuclei was not constant. In some cells nuclei were near the surface membrane, in others they were near the middle, and in yet others they were near the bases. Some cells appeared to possess two or three nuclei, but it is considered that many of the nuclei observed are actually migrating coelomocytes.

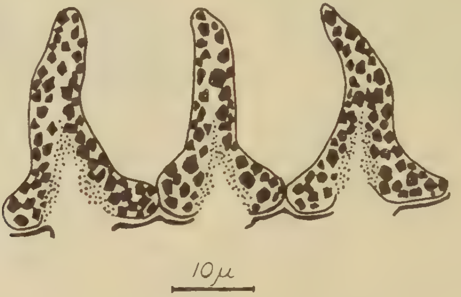

Fig. 7. Three partially split cells of the outer layer viewed in longitudinal section

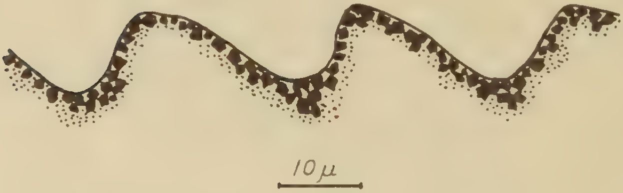

Fig. 8. Two split cells of the outer layer and portions of adjacent split cells all viewed in longitudinal section

(*b*) *Histochemistry*

The granulated compartments and angular bodies found in the cells of the outer layer stained with the basophilic stains eosin, erythrosin, acid fuschin, and orange G. The acid dye chlorazol black E stained them greyish.

Sections fixed in Bouin's fluid were stained for polysaccharides by the histochemical method of Hotchkiss (1948). The outer membrane, the cell boundaries of the cells of the outer layer, and the boundaries of their contained granulated compartments, stained positively, but the angular bodies and the material within the compartments did not stain.

With Alcian blue 8GS and toluidine blue, allegedly specific for 'mucin' (Steedman, 1950) and polysaccharide sulphates (Lison, 1935) respectively, the cells of the outer layer did not stain at all.

However, when Bouin-fixed sections were stained for protein with the mercuric chloride bromphenol blue technique of Mazia, Brewer, and Alfert (1953), the granulated compartments and angular bodies in the cells of the outer layer stained heavily. Positive results were also given by these structures when the histochemical adaptation of the Millon reaction for proteins containing tyrosine (Bensley and Gersh, 1933) was used on similar sections. However, negative results for protein were obtained when the sections were boiled with ninhydrin.

The proteinaceous material was resistant to the action of dilute hydrochloric acid. Sections fixed in Bouin's fluid were heated to 98° with N HCl for 15 min. After this treatment the angular bodies still stained strongly for protein with mercuric chloride / bromphenol blue and with Millon reagent. Likewise, the proteinaceous material of the angular bodies appeared to be resistant to the action of 0·5% pepsin (pH 1·8) to which they were exposed for 24 h. Also it was not visibly affected by alkaline thioglycollate solution, and hence keratin-type proteins containing disulphide bands do not appear to be present (Goddard and Michaelis, 1934).

Guislain (1953) studied the Cuvierian tubules of *H. impatiens* histochemically, and his appears to be the only published work on the chemistry of the tubules. He stated (p. 1256) that he was not able to determine 'la nature exacte des fines granulations fuchsinophiles de la zone externe' and that 'ces grains se sont révélés inertes à l'égard des différents réactifs employés'. Such being the case, the positive reactions for polysaccharides and polysaccharide sulphate given by cells of the 'z. externe' on p. 1255 presumably refers to reactions given by vacuoles which he mentioned were present at the periphery of these cells. Such vacuoles were not observed in *H. leucospilota*. In this species the histochemical tests outlined earlier indicate that the angular bodies and their apparent precursors in the cells of the outer layer contain proteinaceous materials.

(*c*) *Chemistry*

Tubules ejected into distilled water were taken and the outer cells stripped from the inner fibrous core. The material so obtained gave a positive Millon reaction and assumed a deep blue colour when heated with 0·1% ninhydrin indicating the presence of proteinaceous materials.

THE MUSCULATURE

Examination of sections and whole mounts of tubules revealed the disposition of the muscles. In the connective tissue between the inner ends of the cells of the outer layer were longitudinal muscle-fibres. These were about 0·5 μ in diameter and occurred chiefly in parallel bundles, each bundle containing 7–11 fibres. The bundles were approximately 10 μ apart and hence one is found in the connective tissue on either side of each of the longitudinal rows of cells of the outer layer (fig. 6). However, it was noted that at irregular intervals one or more fibres left a bundle at an acute angle and joined up with the fibres of an adjacent bundle. This occurrence accounted for the variation obtained when counts of the number of fibres per bundle were made in different regions of a tubule. In *H. impatiens* 8–15 fibres were found in each longitudinal muscle-bundle (Cuénot, 1891), and possibly the variation in number of fibres per bundle observed in this species is due to a similar occurrence.

The average number of fibres per bundle seems, however, to vary with the species studied. Thus Ludwig and Barthels (1892) found 2–4 in *H. lamperti* Ludwig; 3–7 in *H. poli* Della Chiaje, *H. klunzingeri* Lampert, and *H. pervicax* Selenka; 4–6 in *H. fusco-cinerea* (Jaeger); 7–8 in *H. forskali* Della Chiaje; an average of 8 in *H. marmorata* (Jaeger); and 5–10 in *H. marenzelleri* Ludwig.

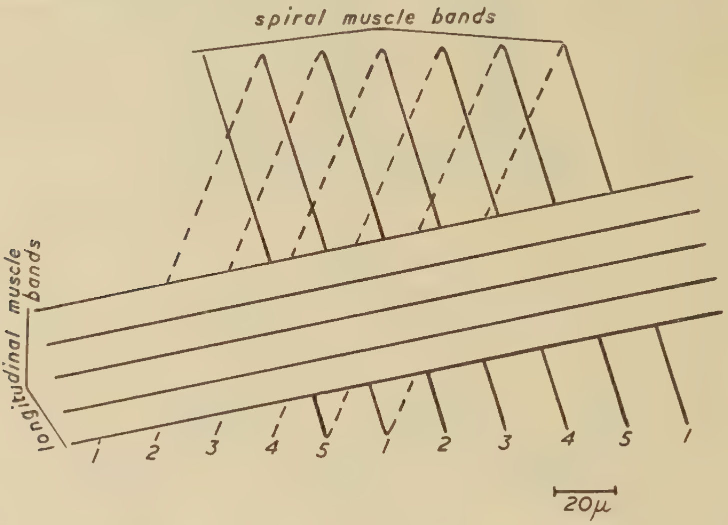

FIG. 9. Diagram showing arrangement of the longitudinal and spiral muscles in a tubule

The longitudinal muscles of *H. leucospilota* were prominent in whole mounts of thick unstretched tubules where their course was observed to be parallel to the long axes of the tubules. In slightly stretched tubules the longitudinal muscles followed the pattern of a loose anticlockwise spiral from the point of origin of each tubule (fig. 7). In whole mounts of greatly stretched tubules the longitudinal muscles could not be seen.

Just internal to the longitudinal muscle-bundles were the spiral muscle-bands. Five separate muscle-bands, running in a clockwise direction from the point of origin of each tubule, could be distinguished (fig. 9). Each band consisted, apparently, of a single thick muscle-fibre about 2 μ in diameter. In whole mounts of slightly stretched tubules these bands were seen to run around each tubule immediately below the furrows occurring between the surface ridges. Hence, there was normally a band on either side of each spiral row of cells of the outer layer (fig. 6). In a slightly stretched tubule 1·8 mm in diameter, adjacent turns of the same spiral muscle-band were about 100 μ apart (fig. 9). When a tubule was stretched the interval between adjacent turns of the same spiral muscle-band increased and the bases of the cells of the outer layer became bent towards a spiral muscle-band. Eventually, the ells split along their long axes and the spiral muscle-bands then run just elow the centres of a spiral row of split cells (fig. 8). This possibly explains he conflicting statements made by some of the earlier authors on the position f the spiral muscle-bands with respect to the overlying cells of the outer ayer.

The existence of separate spiral muscle-bands in the Cuvierian tubules and heir role in causing the splitting of the cells of the outer layer has not been ommented on previously.

Hamann (1883) described the muscle-layer internal to the longitudinal nuscle-bundles as a circular muscle-layer. However, Hérouard (1889) noted hat in *H. forskali* the inner muscle described a spiral around each tubule and similar arrangement has been found in all species but one studied by subequent investigators. The exception is *Actinopyga agassizi* in which Hyman 1955) has found circular muscles.

Jourdan (1883) believed that each 'circular' muscle-band consisted in *H. npatiens* of 3–4 fibres, but Cuénot (1891) maintained that only 2 fibres were resent in each band. Ludwig and Barthels (1892) noted that in numerous ther species only one fibre occurs per band and they pointed out that the isposition of the muscle-fibres is greatly affected by the degree of contraction f the tubules. In contracted tubules adjacent turns of the spiral muscles lay ery close together. As a result these muscles appeared to pursue circular ourses and to consist of more than one muscle-fibre.

Observation of fresh whole mounts of tubules from *H. leucospilota* which ad been placed in a small quantity of coelomic fluid revealed that short egments of each spiral muscle-band were capable of contracting independntly of the rest of the band. Contraction of a segment lessened the diameter f the tubule in its vicinity.

Presumably, the longitudinal and spiral muscles of the tubules are antagoistic. If the longitudinal muscles contract and the spiral muscles relax, the ubules are shortened and thickened, whilst relaxation of the longitudinal nuscles and contraction of the spiral muscles results in elongation and thinning f the tubules. In view of the fact that the spiral muscles are anchored at one nd and free at the other, it would seem that there must inevitably be some wisting of the tubules when these muscles contract. This would account for he observation that in stretched tubules the longitudinal muscles followed he pattern of a loose anticlockwise spiral from the point of origin of each ubule.

THE FIBRES AND INTERFIBRILLAR MATERIAL

a) *Histology*

Observations were made on fibres both in teased preparations of fresh ubules and in longitudinal and transverse sections of tubules. The fibres had strong affinity for chlorazol black E and stained a bright blue with Mallory's nd Heidenhain's 'azan' stains. These stains gave a clear picture of fibre istribution.

The fibres had an average diameter of about 0·4 μ but diameters range from the limit of resolution to about 1·5 μ. Under the phase-contrast microscope the thicker fibres were seen to be aggregates of thinner ones. In n case were the fibres observed to branch.

On cursory examination the fibres seemed to interlace in all planes, bu closer study revealed that the majority ran in undulating sheets whic pursued a spiral course around the axis of each tubule. Even in small newl formed tubules the fibres were oriented in a similar fashion. These observations agree with the findings of Cuénot (1891) and Ludwig and Barthel (1892).

Sections of tubules which had been ejected into sea water prior to fixatio were stained with Mallory's stain and examined. The average diameter of th fibres in these sections did not differ from that of fibres observed in tubule taken directly from the body-cavity of a holothurian. However, the fibre bundles were not coiled as tightly as they were in the latter tubules. I greatly stretched tubules the course followed by the fibres was almost parall with the long axes of the tubules. It would seem that in such cases there ha been a straightening of the spiral fibre-bundles.

Coelomocytes were found frequently amongst the fibres but the interfibrillar ground-mass possessed no obvious structure.

(*b*) *Histochemistry*

The chemistry of the fibres present in the Cuvierian tubules of holothuria does not appear to have been investigated previously. In *H. leucospilota* th fibres stained with the aniline blue component of the polychrome stain Mallory's, Heidenhain's 'azan', and Masson's. With Van Gieson's stain an Weigert's resorcin fuschin stain the fibres stained reddish. The stainin behaviour of the fibres is therefore similar to that exhibited by the collage fibres of vertebrate connective tissue.

Sections of the Cuvierian tubules, fixed in Bouin's fluid, were stained fo proteins using the histochemical technique of Mazia, Brewer, and Alfe (1953). The fibres did not stain. Also negative results were obtained when th sections were boiled with ninhydrin. Sections placed in 0·5% pepsin (pH 1· for 24 h and subsequently examined with the phase-contrast microsco showed the fibres unaltered and comparable in all respects with those i untreated control sections. These particular tests thus failed to indicate tha proteins were present in the fibres.

The presence of polysaccharides was indicated when the fibres in Bouin fixed sections of tubules became reddish-violet after treatment by the meth of Hotchkiss (1948).

Sections of tubules fixed in equal volumes of 8% basic lead acetate an 16% formalin were stained with toluidine blue following the method Sylvén (1941). The fibres became purplish-red, but the interfibrillar groun mass did not stain. Such metachromatic staining is indicative of the presen of highly polymerized carbohydrates which according to Lison (1935) a esterfied with sulphuric acid. Michaelis (1947), however, has shown that polymerized carbohydrates themselves produce metachromatic effects by virtue of their carboxyl groups.

The presence of acid mucopolysaccharides in the fibres was next investigated using Hale's (1946) histochemical method, the specificity of which, however, appears doubtful. Both the fibres and the interfibrillar material stained blue, the fibres intensely so. In view of this positive result it was thought that hyaluronic acid might be present. Sections, some fixed in Bouin's and some in Carnoy's fluids, were incubated for 14 h at 37° C with 0·01% bovine testicular hyaluronidase ('hyalase', a product of Benger's Ltd., was used), washed with distilled water and stained respectively with toluidine blue and by Hale's (1946) method for acid mucopolysaccharides.

With toluidine blue the fibres exhibited metachromasy and they also stained positively for acid polysaccharide, though in neither case as strongly as they did in control sections untreated with hyaluronidase. However, many fibres in the treated sections appeared disarranged and the interfibrillar material did not stain at all. Incubation of sections for 36 h with two changes of hyaluronidase did not result in the removal of polysaccharide material from the fibres.

In an attempt to remove this material, sections of tubules fixed with Bouin's fluid were heated at 98° C with N HCl for 15 min. Subsequently, when washed and stained for polysaccharides by Hotchkiss's method and by toluidine blue, negative results were obtained in both cases. Observation with the phase-contrast microscope revealed that fibres were still present but these were disarrayed. Apparently polysaccharide material had been removed from the fibres and the interfibrillar spaces as a result of heating with hydrochloric acid. When additional sections, similarly treated with N HCl were washed and stained for proteins by the mercuric chloride / bromphenol blue method of Mazia, Brewer, and Alfert, the fibres stained positively though not strongly.

The above histochemical tests indicate that the fibres contain both protein and polysaccharide components. The polysaccharide material is possibly an acid mucopolysaccharide, though not apparently hyaluronic acid, and is loosely linked to the protein component from which it can be removed by mild treatment with hydrochloric acid. Presumably the presence of the polysaccharide material masks or interferes with the histochemical tests for protein used. The fibres themselves seem to be cemented together by an acid mucopolysaccharide which may be hyaluronic acid although this material has not been detected previously in echinoderms (Guislain, 1953).

(c) *Chemistry*

Specimens were allowed to eject tubules into distilled water. Such ejected tubules were not particularly sticky and the cells of the outer layer could be stripped from each tubule. This operation was tedious but resulted in a quantity of material which consisted of the fibrous cores of connective tissue and contained coelomocytes. The latter were cytolysed by washing the fibrous cores repeatedly in distilled water.

Fibrous cores, heated in distilled water, swelled initially but at a temperature of 63° C contracted suddenly to about a third of their original length. Prolonged heating in water at 100° C caused them to dissolve. They swelled in dilute acids and alkalis, dissolved slowly in concentrated alkalis and acids (except H_2SO_4 where solution was immediate), and were only slowly attacked by pepsin (0·1% at a pH of 1·2). Thus the behaviour of the fibrillar material is typical of that exhibited by the protein collagen.

Fibrous cores, after thorough washing, were heated at 98° C with N HCl in stoppered test tubes for 1 h. The fluid in each case was then poured off into a crucible. This was placed in a desiccator over solid NaOH and the desiccator evacuated. After the fluid was removed, a slight brownish deposit remained in the crucible. This was dissolved in a little water and tested for the presence of hexosamine following the method of Elson and Morgan (1933). A positive reaction was obtained.

It was concluded in view of the above reactions and the histochemical tests described previously that the connective tissue of the tubules consisted of collagen fibres associated with a polysaccharide which is, in part at least, mucopolysaccharide.

THE CENTRAL CORE

The core is comprised of packed cells which appear to be identical with certain morula-shaped coelomocytes observed in the coelomic fluid. The coelomocytes are surrounded directly by the fibrillar matrix into the interstices of which they frequently pass.

Sections of tubules which had been ejected into sea water prior to fixation were studied and it was noted that in each case the cells of the central core had been pushed against the bounding fibrillar matrix and a central cavity ran the length of each tubule. This finding is consistent with the view of Mines (1912) who maintained that water was forced into the tubules when they were discharged.

THE REGION OF ATTACHMENT OF THE CUVIERIAN TUBULES

The tubules arise from papillae on the walls of the base of the left respiratory tree and on the common stem of the respiratory trees (fig. 1). One, two, or three tubules were observed to arise from each papilla. Both the papillae and the walls of the respiratory trees contained rod spicules and reddish pigment cells.

'Azan'-stained serial sections passing through the stem of the respiratory trees and through the papillae were studied. It was found that the lumen of the respiratory trees possessed an epithelium of vacuolated cells, about 40 μ high, the boundaries of which were indistinct because of the presence of enormous numbers of small coelomocytes. Underlying this epithelium was a fibrillar matrix, the fibres of which increased in thickness towards the periphery. This fibrillar matrix was permeated with coelomocytes of varying sizes. Two muscle-layers—a circular and a longitudinal layer—formed the outer boundary of the fibrillar material, and, external to these, was the coelomic epithelium.

The papillae from which the tubules arose were seen to be evaginations of the wall of the respiratory trees. At their point of origin the papillae averaged about 360 μ in diameter but they tapered to a diameter of about 120–30 μ at their junction with the tubules. Here the coelomic epithelium covering the papillae gave rise to the cells of the outer layer of the tubules and the muscle-layers of the papillae gave rise to the muscles of the tubules. The connective tissue of the tubules was continuous with that of the respiratory trees but in the tubules the fibres were coarser and stained much more strongly with aniline blue.

Extensions of the lumen of the respiratory trees were present in the papillae. However, in each papilla the lumen ended blindly at the base of the tubule or bases of the tubules. Normally, therefore, sea water cannot pass into the tubules.

At the junction of the tubules with the papillae, rings of muscle surrounded by lacunae were observed. These muscles are believed to be responsible for causing the tubules to break away from the papillae when the tubules are ejected (Hérouard, 1889).

DISCUSSION

Histologically, the Cuvierian tubules are difficult to interpret. The factor chiefly responsible for the confusing pictures presented by many sections is the arrangement of the muscle-layers. Upon the degree of contraction exhibited by the longitudinal and spiral muscles, depends the disposition and, in the case of *H. leucospilota* at least, the structural integrity of the outermost layer of cells, the orientation and degree of compaction of the bundles of connective tissue and the degree of coiling of the central core. Also, coelomocytes present in the tubules are frequently cytolysed during preparation of sections. Then, too, it would appear that there is variation in tubule structure and in coelomocyte types from one species to another. Consequently, there has not been unanimity of opinion regarding several aspects of the histology of the tubules.

In one feature the tubules of *H. leucospilota* differ markedly from those of other holothurian species so far investigated. Most workers who have examined holothurian Cuvierian tubules have observed the presence of an axial lumen surrounded by gland cells. No such lumen or gland cells were observed in the tubules of *H. leucospilota*.

Also, although structures which would correspond with the granulated compartments and angular bodies found in the outer cells of the tubules of this species have been noted in the tubules of other species, the observation that in some cells granulated compartments and angular bodies coexist has not been made previously.

The splitting of the cells of the outer layer when the tubules are stretched, as observed in *H. leucospilota*, has also not been commented on by previous investigators but there is the possibility that they have misinterpreted this splitting of the cells as noted earlier.

In other respects the general structure of the tubules of *H. leucospilota* conforms with that of the tubules of most other species studied. Thus the orientation of the fibres, the general arrangement of the musculature and the general disposition of the cells of the outer layer seem to follow a fundamental pattern.

The finding that the angular bodies and their apparent precursors in *H. leucospilota* were composed of proteinaceous material was of interest but the chemical constitution of this material and the reason it acquires adhesive properties when brought into contact with sea water await investigation.

Several attempts have been made to explain the method of discharge and mode of elongation of the tubules. Jourdan (1883) considered that an unrolling of the bundles of connective tissue within the tubules was primarily responsible for their elongation. Hérouard (1889) noted that in *H. catanensis* Grube each tubule has, at its base, a sphincter muscle which surrounds an orifice opening into lumen of the respiratory trees. Contraction of the respiratory trees, he believed, results in forcible injection of water into the tubules causing them to elongate. This view was supported by Mines (1912) who brought about elongation of the tubules of *H. nigra* Peach by injecting sea water into their lumina.

Minchin (1892), however, noted that a tubule will continue to elongate after it has broken away from the body of a holothurian. Also he noted that undischarged Cuvierian tubules of *H. nigra*, after removal from the animal's body, would elongate if placed in sea water. He ascribed their elongation to some intrinsic activity of the tubules.

Cuénot (1891) attributed the elongation of the tubules within the body-cavity and their subsequent ejection to injection of water but, after their ejection, he believed that the spirally wound muscles and connective tissue-fibres unroll so that the tubules elongate and at the same time the external epithelium acquires adhesive powers.

Observations made on the tubules of *H. leucospilota* support Cuénot's ideas. It was noted that in sections of ejected tubules the cells of the central core were scattered and pushed against the surrounding fibres and that the space they had occupied was transformed into a cavity. This is consistent with the probable effect of forcible injection of water into the tubules. The injected water must be derived from the water present in the lumen of the respiratory trees. In order to gain entrance to the tubules the water must burst through the blind ends of the extensions of the lumen of the respiratory trees found in the papillae. Consequently, it is necessary for this water to be under pressure. This pressure would be developed when the longitudinal muscles in the respiratory trees and the cloacal muscles contracted. The water, however, can remain under pressure only whilst the anus remains closed. It follows that water must be forced into the tubules before they are ejected via the anus.

Ejection occurs when the cloacal wall tears. Since the tubules are normally flaccid structures it is possible that if they were not stiffened by the injection of water prior to ejection they would entangle one another and obstruct the rent in the cloacal wall through which they must pass.

The observation that isolated tubules will swell if placed in sea water suggests that the injection of tubules with water is not the sole factor responsible for their elongation. Possibly the two muscular systems present in the tubules are involved in this process but the mode of action of these muscle systems is not clear. The role played by the helically arranged bundles of connective tissue in the elongation process is uncertain, but since the fibres of the connective tissue present in *H. leucospilota* are collagenous and since collagen fibres do not possess elastic properties, it would seem that these fibres could not be actively involved in this process.

As the tubules lengthen, the cells of the outer layer are torn open exposing the proteinaceous angular bodies which form an adhesive layer upon contact with sea water. This layer sticks to most objects brought into contact with it but not to the holothurian body-wall.

Release of the ejected tubules from their attachments at the papillae appears to be accomplished by contraction of rings of muscle in this narrow region.

The finding that the fibres present in the tubules of *H. leucospilota* are collagenous was not surprising since X-ray diffraction studies (Marks *et al.*, 1949; Randall *et al.*, 1952) have previously revealed the presence of collagen fibres in the connective tissue of echinoderms.

However, the collagen fibres of the tubules of *H. leucospilota* are associated with acid polysaccharides which appear to be, in part at least, mucopolysaccharides. Also, the interfibrillar ground-mass contains a polysaccharide which is removed by testicular hyaluronidase. These findings are noteworthy because in vertebrate connective tissues collagen fibres are present and these are always associated with a mucopolysaccharide, chondroitin sulphuric acid, and, in the interfibrillar groundmass, hyaluronic acid occurs.

It is known that the collagen fibres of vertebrates are associated with and produced by cells called fibroblasts. By analogy it might be expected that cells with a similar role would exist amongst the collagen fibres of holothurians. Morula-shaped cells are found amongst the collagen fibres in the Cuvierian tubules of *H. leucospilota* and their role will be investigated. It might perhaps be significant that cells with a similar appearance are responsible for producing the tunicin fibres of the test of the ascidian, *Pyura stolonifera* (Heller) (Endean, 1955 *a*, *b*, *c*).

In the tubules of the holothurian studied collagen fibres form the major structural components. The ability of collagen fibres to resist tensile forces enhances the effectiveness of the tubules as organs of defence.

The author is indebted to Professor W. Stephenson of the University of Queensland for continued interest and helpful advice and to Dr. M. C. Bleakly of the University of Queensland for reading and discussing the manuscript. The technical assistance given by Mr. J. Jenkins and Mr. C Illidge is gratefully acknowledged. Enlargements of the author's negative and photographs of the author's drawings were prepared by Mr. E. Hollywoo of the Photographic Department, University of Queensland. The autho wishes to thank the Great Barrier Reef Committee for providing facilities a Heron Island.

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Some Aspects of the Structure and Cytology of the Gills of *Lampetra fluviatilis*

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With one plate (fig. 6)

SUMMARY

A description is given of the structure and vascularization of the lampern gill.

A comparison of the cellular components of the gill epithelium taken from lamperns at various stages of their spawning migration show that certain types of cells are common to all animals. These include the cells covering the gill platelets, the mucous cells, and certain basal cells from which others originate. The mucous cells are quite different in structure from those of teleosts.

There is a progressive loss of large acidophilic cells from certain regions of the gill filament as animals enter fresh water during the earlier stages of the spawning migration. These cells are similar in both structure and location to those described as chloride excretory cells from the gills of marine teleosts. They are typically large, flask-shaped cells with basal nuclei and they are filled with rod-shaped or spherical mitochondria. It is believed that they are responsible for the extra-renal excretion of monovalent ions during the process of marine osmoregulation in the lampern. The chloride excretory cells are very numerous in a limited number of fresh-run animals which are able to osmoregulate in 50% sea water.

In the normal course of events the chloride excretory cells are replaced by a smaller type of cell which is also rich in mitochondria, but unlike the excretory cells these lie at the surface of a transitional epithelium. It is argued that these cells may be responsible for ion uptake from fresh water in the maturing animal.

In male lamperns, large glandular cells of unknown function appear in the gill epithelium as the animal nears the time of spawning.

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INTRODUCTION

THE osmoregulatory ability of the lampern (*Lampetra fluviatilis* L.) changes as the animal passes into fresh water during the early stages of its spawning migration (Morris, 1956). At this time, fresh-run animals exhibit varying degrees of osmoregulatory ability in 33% sea water; an environment which is slightly hypertonic to their blood. Some fresh-run animals are able

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to keep the osmotic pressures of their blood below that of the environment by a similar mechanism to that already known for marine teleosts (Morris, unpublished). Other fresh-run animals are unable to osmoregulate at all and can only live by tolerating the raised osmotic pressure caused by immersion in dilute seawater solutions. In this respect they show similar responses to maturing animals which have lived in fresh water for 2 or 3 months. Many fresh-run animals show responses which are intermediate between these two extremes and it is held that these represent stages in the breakdown of the marine mechanism of osmoregulation as the animal establishes itself in fresh water and develops a new mechanism.

In marine teleosts, water lost as a consequence of osmotic conditions is replaced by swallowed sea water. Water and monovalent ions are absorbed preferentially from the gut into the blood-stream and ion balance is then restored by the continuous extra-renal excretion of monovalent ions and also by the renal excretion of excess divalent ions (Smith, 1930). The gills are responsible for extra-renal excretion (Smith, 1930; Keys, 1931 *a*, *b*; Schlieper, 1933) and it was in this tissue that Keys and Willmer (1932) first discovered large acidophil cells which were found to be closely associated with the afferent branchial circulatory system. They believed that these cells were responsible for the active excretion of large amounts of monovalent ions which resulted from the processes of osmoregulation and therefore named them 'chloride cells'. Bevelander (1935 and 1936) maintained that the cells described by Keys and Willmer were stages in the production of mucous cells; a view which is difficult to reconcile with the more recent work of Liu (1942, 1944), Copeland (1948), Pettengill and Copeland (1948), Burns and Copeland (1950), and Getman (1950), whose findings support the original hypothesis.

The present studies on gill epithelia were undertaken to see whether similar cells exist in the gills of the lampern and, in view of the change of the osmoregulating ability of the lampern with time, whether there was any corresponding change in the nature of the gill epithelium.

MATERIALS AND METHODS

Lamperns were obtained from the River Trent. They were transported to Nottingham in wooden boxes containing damp grass and were kept successfully in a large aquarium tank provided with running water. The lamperns immersed in diluted sea water were graded into osmoregulating and non-osmoregulating animals by means of methods described elsewhere (Morris, 1956).

Eels were obtained from the lake in University Park, Nottingham, by kind permission of the Trent River Board. Only large specimens were used and those which were immersed in sea water were left in this environment for a minimum period of 4 days. Gill material from conger eels and sea bream, which were also used for comparison with the lampern, was obtained from Plymouth.

Whole gill pouches were dissected out from lamperns and fixed, whilst maller pieces of gill material were taken from marine teleosts. Micro-natomical work was conducted mainly on Susa-fixed material stained by the zan technique. Helly's fluid, followed by incubation in 3% potassium ichromate for 3 days at 37° C served as a general cytological fixative and also o preserve mitochondria and phospholipid material within cells. Sections ut from this material were stained by the Kull method for mitochondria Baker, 1945) and the Sudan black technique for demonstrating phospholipid Thomas, 1948). Toluidene blue and thionine were used to demonstrate the resence of mucous and mucous-producing cells by their metachromic ction. Sections were immersed in a 0·2% aqueous solution, blotted dry ith filter paper, then passed quickly through acetone to xylene and ounted in balsam. Frozen-dried material was also employed for cyto-gical purposes. The conventional method of freezing-drying was used uring which small pieces of gill material were immersed in iso-pentane hilled to −165° C in liquid air. They were then transferred to a freezing-rying apparatus of similar design to that employed by Packer and Scott (1942) nd maintained at −70° C for 3 or 4 days in a vacuum of 10^{-5} mm of mercury. fter this time, the tissue was slowly allowed to attain ambient temperature nder reduced vacuum and later embedded in water-free paraffin wax. ections of material prepared in this way were fixed to slides by a smear of lbumen and flattened, after warming, by slight pressure from the thumb. he paraffin wax was removed by xylene or petrol-ether and the sections were hen denatured in alcohol for an hour or more. Most of the usual staining ethods gave unsatisfactory results after this treatment, and by far the best esults were obtained with Heidenhain's iron haematoxylin.

THE MICRO-ANATOMY OF THE GILL POUCH

Each of the seven pairs of gill pouches of the lampern is lenticular in shape hen contracted and is composed of an anterior and posterior plate, each onsisting of 17 or more parallel filaments (fig. 1). The plates, which are oughly circular, join at their edges to form the complete gill pouch. The gill laments resemble those described for teleosts (Bevelander, 1935; Keys and illmer, 1932.) They are long, curved structures, blade-like in form, and hey taper apically. The broadest surfaces of the filaments oppose one another nd bear numerous thin flat platelets (100 or more) which lie across the long xis of the filament. The filaments join basally near the respiratory canal ituated beneath the oesophagus) and they extend horizontally toward the xternal opening of the gill pouch. Connective tissue binds neighbouring laments along their outer edges and above this, a thin layer of oblique uscle surrounds the pouch.

The blood circulation is also similar in many respects to that described for e gills of teleosts (Bevelander, 1935). Afferent branchial vessels arise from ach side of the divided ventral aorta (fig. 1). These pass between neighbouring airs of gill pouches and branch to supply the posterior plate of one pouch and the anterior plate of the succeeding pouch. The afferent branchial artery supplying the posterior plate separates into numerous vessels which join the afferent filamentar vessels of the more centrally placed filaments about half-way along their length. The peripheral filaments on either side of this region are sometimes supplied by single transverse vessels which join each of the afferent filamentar vessels to one another. In the anterior plate, the afferent branchial artery crosses the base of the filaments on the outside of the gill pouch and gives rise to the afferent filamentar vessels. Blood from each filament is collected by the efferent filamentar vessels, but on the opposit and inner edge of the filaments. Each of the efferent vessels join at the base of the filaments on the inside of the pouch and the vessels from the anterior and posterior plates of neighbouring pouches enter the dorsal aorta as th efferent branchial vessel.

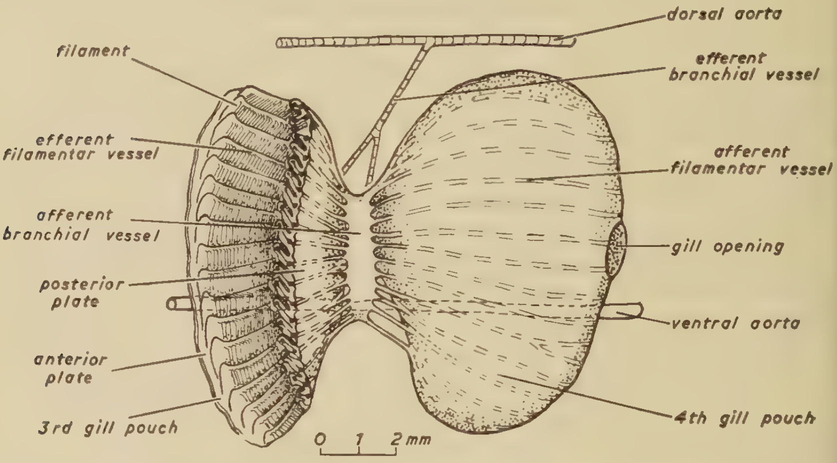

Fig. 1. Diagram of the 3rd and 4th gill pouches of an adult lampern. Half of the posterior plate of the 3rd gill pouch has been removed to illustrate the internal arrangement

Histologically, each gill filament consists of a central core of connectiv tissue surrounding the afferent and efferent blood-vessel. Minute blood channels with thin walls of connective tissue emanate from these into th central blade of the filament. Similar channels are also present in the gi platelets where they form a plexus. The connective tissue channels are sup ported by pilaster cells; small, pillar-like cells with dense nuclei (fig. 2). Th afferent filamentar vessel contains a mass of glandular epithelial cells of un known function and often this appears to divide the vessel into two channels The same tissue also occurs to a lesser extent in the smaller efferent vesse The whole of that part of the filament which lies inside the gill pouch i covered by a layer of epithelial tissue and this varies in character accordin to its location and the length of time the animal has been in fresh water.

THE CYTOLOGY OF THE GILL EPITHELIUM

Comparative studies were made on the gills of fresh-run maturing and mature lamperns taken from animals living in fresh water and from experiments involving immersion in diluted sea water. In all cases, sections were cut parallel to the plates of filaments starting from the outside of the gill pouch. The first 10 or 15 sections were discarded, so that those actually examined contained longitudinal sections of the filaments whose depth varied because of the curvature of the gill plate in one direction and the curvature of the filaments in another. Sections cut in this way thus showed a gradation of different localities which could be studied from the same slide.

The gill epithelia of fresh-run lamperns

These animals showed the greatest variation in the cell types found in gill epithelia. Five main types of cells were recognized and of these, three types (the platelet cells, the basal cells, and mucous cells) were present in all the 40 animals studied.

The *platelet cells* (fig. 2) cover the outer surface of the gill platelets in a single layer which lies on the basement membrane of connective tissue separating the cells from the underlying blood plexus. These, the least variable of all the cells in the lampern gill, are long, flattened structures containing an elongate nucleus. The homogeneous cytoplasm usually contains a variable number of small rods and granules, which from their appearance and staining reactions are probably mitochondria.

The remaining cells of the gill epithelium are found in one of two positions; the epithelium lying between the base and sides of the filaments (the interfilamentar areas), and also the regions between the gill platelets (the interplatelet areas). In both of these positions, the gill epithelium is much thicker than that which covers the platelets and often takes the form of a transitional epithelium which covers the connective tissue surrounding the vascular system. The basal layers appear to give rise to some of the more specialized epithelial cells which form the uppermost layers. The *basal cells* (figs. 2, 3, 4, 6) in these regions are small, round cells with comparatively large, homogeneous nuclei which usually stain heavily and contain one or two large nucleoli. The amount of cytoplasm is small compared with the nuclear size in the lower layers of cells but increases considerably in the upper layers. The cytoplasmic inclusions, though variable in type and distribution are usually present in the outer layers of the basal cells in the form of spheroids of sudanophil material or as short, rod-like mitochondria.

The remaining cells of the gill epithelium have free borders in the interfilamentar and interplatelet areas. Some lie at the surface of a transitional epithelium of basal cells, whilst others extend from a single layer of basal cells covering the vascular connective tissue of the gill filament to the external surface of the gill epithelium.

In some fresh-run animals, the interplatelet area of the gill filament is almost wholly occupied by large cells (15–30μ) which, although they may be round or flask-shaped, are most often represented by long columnar cells which taper apically. Because these cells are similar in many respects to those described as *chloride excretory cells* in the gills of some teleosts, the same

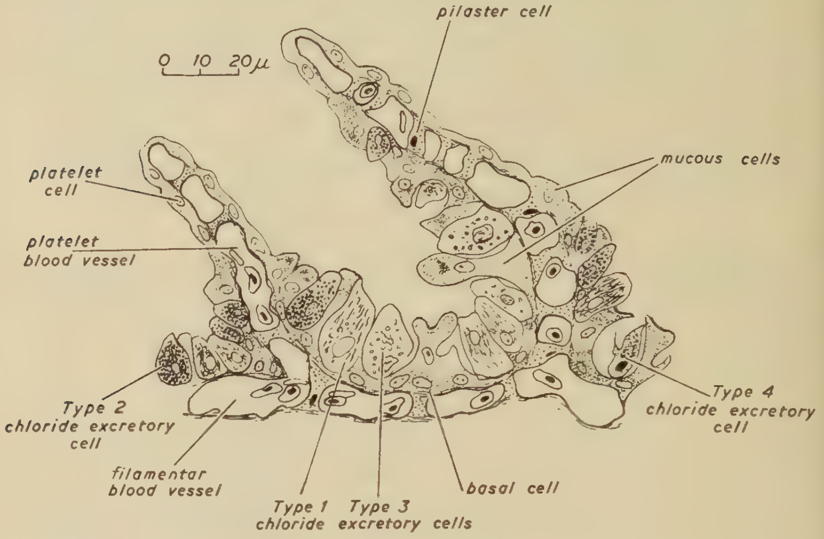

FIG. 2. Cell types from the interplatelet areas of the gills of some fresh-run lamperns

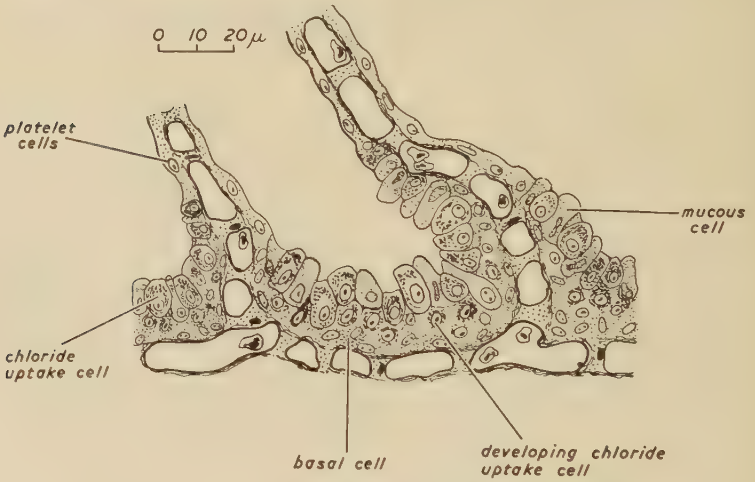

FIG. 3. Cell types from the interplatelet areas of the gills of some fresh-run lamperns, maturing lamperns, and mature females

name has been used in the descriptions which follow (figs. 2, 6, A). The chloride excretory cells of the lampern occur in groups in which the cells are adjacent to one another. They rest on the connective tissue surrounding the vascular system and are separated in this region by small basal cells. In what are judged to be the functional stages of the cell (fig. 2, types 1 and 2), the nuclei are large and usually occupy a basal or central position. The cytoplasm is clear and homogeneous and gives the acidophilic reaction which is characteristic of teleost chloride excretory cells. Large numbers of fuchsinophil inclusions occupy the cells except for a clear apical border. The type 1 cells (figs. 2, 6, A) contain long, filamentous mitochondria which lie parallel and perpendicular to the apex of the cell. The nuclei of this type of cell usually give a faint staining reaction. In type 2 cells (figs. 2, 6, A) the mitochondria are broken up into uniform spheres which vary in their capacity to take up fuchsin and the nuclei contain a sparse network of sharply stained chromatin. In both types of cells the cytoplasm and inclusions are more markedly sudanophil than the surrounding cells; a fact which enables chloride cells to be identified readily. Large spheroids are often obvious in material treated in this way.

Animals taken directly from fresh water showed varying stages in the breakdown and disappearance of the chloride excretory cells. The first signs of breakdown are disorganization of the normal mitochondrial pattern, irregularities in the shape of the nucleus, and vaccuolation of the cytoplasm (fig. 2, type 3). At a later stage, the inclusions disappear, the nucleus becomes pycnotic, and the cell shrinks away from the surrounding tissues (fig. 2, type 4). Finally, the cell disintegrates completely leaving a space in the gill epithelium. The number of regressing cells varied from individual to individual.

After animals had been immersed in dilute sea water (2–3 days in 33% sea water, 1 day in 50% sea water) there was evidence of increased activity on the part of the chloride cells, particularly in those animals which were known to be capable of osmoregulation. In these lamperns, cells of type 1 and 2 predominated. Additional cells appeared to originate from the basal cells of the interplatelet region where there were signs of cell division taking place, and the cytoplasm of many of these cells contained spheroids coloured by Sudan black. In later stages of development, mitochondrial material was present as long rods and frequently the basal cells appeared to merge into small-sized chloride cells. A few of the disintegrating cells (types 3 and 4) were also present. It is difficult to decide whether these were present as the result of cyclical replacement of the excretory cells or as relics of the process of excretory cell loss so evident in many animals taken from fresh water.

Observations on serial sections taken from the gills of seawater animals containing large numbers of chloride cells showed that their filamentar distribution is very similar to that described by Keys and Willmer (1932) for teleosts. The cells are present in the greatest numbers on the afferent side of each of the filaments and extend from here to the lateral and basal epithelia joining each filament. The numbers of cells become smaller toward the efferent vessel of each filament and also toward the tips of the filaments in the region of the afferent filamentar vessel.

Numerous *mucous cells* (figs. 2, 3, 4) also occupy the interplatelet area and these often surround and separate the chloride excretory cells. In this region, the mucous cells are long, columnar structures which are frequently enlarged at their apices (fig. 2). Cells of similar function are also found in the interfilamentar areas of the gills and on the surface of the epithelial tissues surrounding the afferent artery. Here, the cells are small, cubical, and are generally present in layers 4 or 5 cells deep. All mucous cells contain a large, basal nucleus which is oval and well-defined, in which the nucleolus is single and the nucleoplasm homogeneous. One of the most striking characteristics of the mucous cells is a striated border which opens to the free surface of the cell. Mitochondria appear to be absent and cells stain densely with fuchsin. Occasionally large bodies can be seen which take up fuchsin and Sudan black to a small extent. Usually the supranuclear region of the cell appears vacuolated and small spheroids and granules occur in this region also. Sections stained with toluidene blue show a distinct metachromasia in the area above the nucleus and this extends to the striated border and envelopes the surface of the cell. The mucous cells from different localities in the same animal give quite a variable response to toluidene blue, some showing little sign of mucous secretion other than a faint reaction on the surfaces of the cells.

Some fresh-run animals exhibit a cell type (*chloride uptake cells*) which, whilst resembling the mucous cells in size, in the possession of a somewhat fainter striated border and in the position of the nucleus, generally differ in their cytoplasmic inclusions and, to a lesser extent, in the positions they occupy (figs. 3, 4, 6, B). These cells are generally found in the interplatelet area of the filament where they occur as a single layer on the outer edge of a transitional epithelium and only very rarely occur amongst the mucous cells of the interfilamentar area. They are most easily distinguished from the mucous cells by their cytoplasmic inclusions, since they are usually packed with granular and rod-like fuchsinophil structures which simulate mitochondria. Occasionally, larger spheroids can be seen which colour peripherally with Sudan black and stain only faintly with fuchsin. Often, when these cells are present in large numbers, the layers of basal cells below them also contain mitochondria, the numbers of which decrease in the lower layers of cells.

The 'chloride uptake cells' thus occupy similar positions on the gill filament to the chloride excretory cells and both seem to originate from basal cells lying on the surface of the tissue surrounding the blood-vessels of the filaments. They also differ from each other in several important respects. The 'uptake cells' are found only at the surface of a number of layers of cells, they are much smaller and often show a faint striated border. Their mitochondrial content is somewhat sparser than that of the larger cells and is usually distributed in such a way as to leave a clear area of cytoplasm surrounding the nucleus. In the excretory cells, the mitochondria appear to be equally distributed throughout the cytoplasm except for a clear border at the apex of the cell. The mitochondria in this case may take the form of long filaments or spheres in contrast to the smaller rods or granules which are characteristic of the chloride uptake cells. 'Uptake cells' show no signs of the degenerative process which can be seen in the excretory cells.

he gill epithelia of maturing and mature lamperns

The gill epithelia from animals killed after they had been kept in fresh ater for 2–3 months (March) showed similar cell types to those noted in the inority of fresh-run animals, that is, 'chloride uptake cells' lying on the urface of a transitional epithelium. The basal cells were all heavily vacuolated n their peripheral cytoplasm.

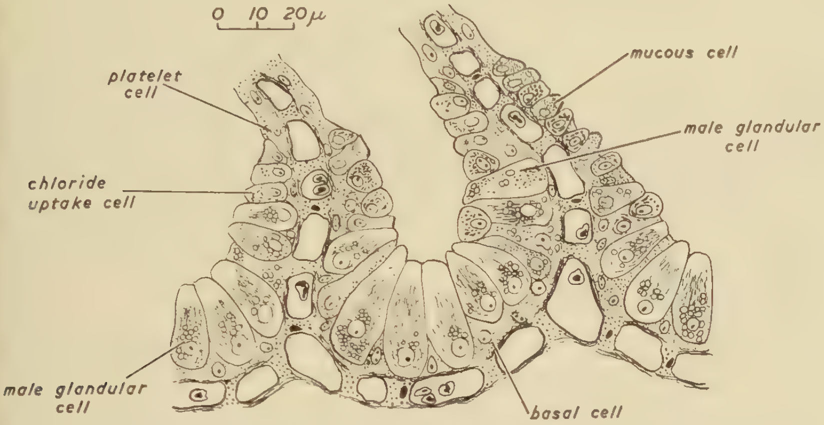

FIG. 4. Cell types from the interplatelet areas of the gills of mature male lamperns

The condition of the gill epithelium in four mature female lamperns was e same as that described for maturing individuals, but in the case of three ales which were examined at the same time, the interplatelet regions were lled with a single layer of large columnar cells (*male glandular cells*) conaining a well-defined nucleus which was generally granular and basal in osition (figs. 4, 6, c). Above the nucleus in these cells, an area of clear acuoles showed heavily stained fuchsinophil, rod-like granules between and n the walls of the vacuoles. The remaining portion of the cells, with the xception of a clear unstained border of cytoplasm, contained long, fine itochondria lying parallel to the long axis of the cell and these were sharply efined against a background of clear cytoplasm. Some of the vacuoles seen rom Kull preparations appear to coincide with spheroids which colour eripherally with Sudan black. Other vacuoles, which had no affinity for udan black, showed a lightly coloured condensate limited to certain parts of he walls of the vacuoles.

The gill tissue from fourteen maturing animals which had been immersed n 33% sea water for periods of time varying from 18–96 h showed no ifference from the condition described for maturing animals taken from resh water, and two mature males and two mature females immersed in the ame external medium also showed no changes from the conditions previously escribed.

The relative numbers of different cell types

An attempt has been made to carry out a rough quantitative survey of the types of cells occurring in the gills of lamperns taken from fresh water at different times during the spawning migration. The experiments extended over two consecutive seasons and during each season, three different groups of animals were used (fresh-run, maturing, and mature animals). The individuals comprising a group were killed at the same time. Platelet cells and mucous cells were found to be present in all the sections of gill pouches which were examined, whilst the relative proportions of basal cells, chloride excretory cells, 'chloride uptake cells',and male glandular cells appeared to vary according to the length of time the animal had been in fresh water.

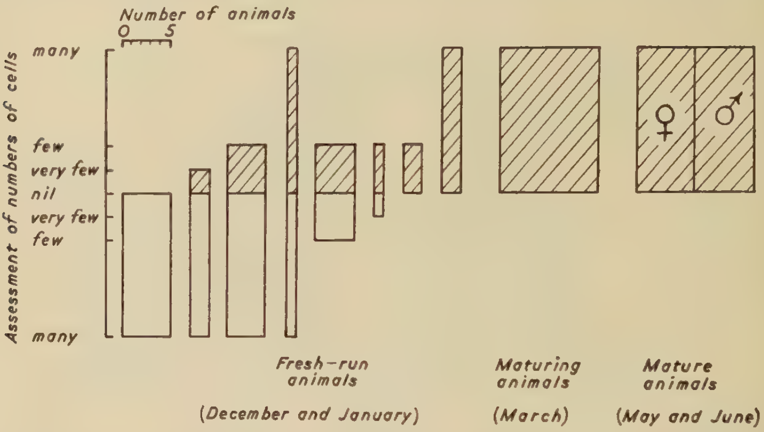

FIG. 5. The distribution of some of the different types of secretory cells in the lampern gills taken from animals at various stages of their spawning migration in fresh water. Unshaded areas: 'Chloride output cells'. Shaded areas: 'chloride uptake cells'. Mature male animals have male glandular cells in addition to 'Chloride uptake cells'

The results of the survey are summarizd in fig. 5. The relative numbers of chloride excretory cells, 'chloride uptake cells' and male glandular cells were assessed by grading the number of cells of one type into the categories 'many', 'few', 'very few', and 'nil'. No attempt was made to count the cells because it was almost impossible to obtain strictly comparable sections owing to the difference of curvature of the gill pouches taken from individuals. In the case of fresh-run animals, similar assessments have been grouped together and ranked according to the relative numbers of excretory or 'uptake' cells.

The survey shows that fresh-run animals vary considerably in the component cells of their gill epithelia. The way in which the excretory and 'uptake' cells are distributed suggests that one cell type is replacing the other and this information, coupled with the fact that the gills of maturing and mature animals contain only 'chloride uptake cells', is in accordance with the view that the 'chloride uptake' type of cell completely replaces the excretory cells.

As this replacement occurs, a change in the distribution of basal cells also takes place. The presence of abundant chloride excretory cells is associated with relatively few basal cells (fig. 2), whilst 'chloride uptake cells' are accompanied by many more basal cells which take the form of a transitional epithelium giving rise to 'uptake cells' at the surface (fig. 3). The male glandular cells in mature males occupy most of the interplatelet region of the gill filaments, displacing the chloride uptake cells to the lower regions of the gill platelets. Basal cells are less numerous in this instance (fig. 4).

DISCUSSION

The large secretory cells observed in the gills of some fresh-run lamperns are similar to those described by Keys and Willmer (1932), Liu (1942, 1944), Copeland (1948),and Getman (1950) from teleosts gills. Here, they are thought to be responsible for the extra-renal excretion monovalent ions during marine osmoregulation. A direct comparison of lampern cells with those of representative marine teleosts (*Anguilla vulgaris*, the conger eel and the sea bream) showed that these cells agree in shape, size, and distribution and moreover, show similarities in their inclusions. In both teleost and lampern material the nuclei are basal, whilst high mitochondrial content, general sudanophilia, and the presence of spheroids covered by a layer of sudanophil material are also common features. The chloride excretory cells of the lampern and those of the teleosts studied contained neither an apical vacuole such as that described by Copeland (1984) in the chloride cells of *Fundulus*, nor an apical pit like that seen in the chloride cells of *Anguilla rostrata* by Getman (1950).

A number of observations, apart from those showing the obvious structural similarities with teleost chloride excretory cells, support the conclusion that the cells play an important part in the process of marine osmoregulation in the lampern. The chloride excretory cells studied from the gills of those fresh-run animals capable of osmoregulation in 50% sea water were very numerous and appeared to be undergoing a cyclical process of development from the underlying basal cells. The cells of these animals frequently showed thread-like mitochondria orientated perpendicular to the long axis of the cell, suggesting that an active process was taking place. Moreover, in fresh-run animals taken from fresh water, the number of chloride excretory cells varied from individual to individual due to a progressive loss of these cells (fig. 5). This process takes place at a time when the osmotic response is very variable (Morris, 1956) and suggests that the loss of these cells may be partly responsible for the breakdown of the marine osmoregulating mechanism. Finally, it should be noted that the excretory cells are entirely absent in maturing and mature animals which are normally restricted to fresh water. These animals are unable to osmoregulate in environments which are mildly hypertonic to their blood (33% sea water) although they can tolerate them for some time (Morris, 1956). Even after 5 or 6 days in 33% sea water, none of the gills examined from maturing individuals showed signs of developing chloride excretory cells and one must conclude that the basal cells lose their competence to produce new excretory cells soon after the animals enter fresh water.

The argument put forward by Bevelander (1935), that chloride cells are really stages in the production of mucous cells, is untenable on the evidence available from teleost material (Copeland, 1948; Getman, 1950) and observations from the lampern only serve as additional confirmation. In this animal, chloride excretory cells do not give a mucous staining reaction with toluidene blue, though mucous cells react. Moreover, the mucous cells appear to be of a completely different type from those described for teleosts (Bevelander, 1935). They are smaller, possess a striated border and a well-defined nucleus and contrast sharply with the large homogeneous cell of teleosts which is usually enucleate in the secretory phase. It seems very unlikely that the chloride excretory cells, which are so similar in the two groups, could be related functionally to two completely different types of mucous cell.

Pettengill and Copeland (1948) have suggested that the chloride excretory cells of *Fundulus heteroclitus* may also be responsible for the active uptake of ions in fresh water. This process is known to be one of wide occurrence in freshwater teleosts and has been shown to be localized in the gill region (Krogh, 1939). The lampern also takes up ions actively during the freshwater phase of its life-history (Wikgren, 1953; Morris, unpublished), but, because chloride excretory cells are absent during the major part of the time that the animal spends in fresh water, the cellular mechanism is certain to be of a different type from that suggested for euryhaline teleosts. The chloride excretory cells in the lampern are replaced by a smaller cell type, rich in mitochondria and therefore presumably active metabolically (Green, 1952). These cells appear at a time when the animal is known to be taking up ions from fresh water, and it therefore seems very probable that these cells are responsible for the uptake. It is for this reason that they have been named 'chloride uptake cells'.

The functions of the male glandular cells are unknown. These cells appear only in sexually mature males alongside the 'chloride uptake cells'. Their structure and occurrence suggests that they may secrete some substance of sexual significance, presumably connected with spawning.

FIG. 6 (plate). Sections of lampern gill pouches along a gill filament to show cells in the interplatelet areas. In each case the main axis of the filament is horizontal and the platelets rise vertically from this.

A, chloride excretory cells from an animal osmoregulating in 50% sea water. Small dark basal cells can be seen on the left under the large excretory cells. (Frozen-dried, Heidenhain's haematoxylin.)

B, 'Chloride uptake cells' from a maturing animal taken from fresh water. Note the basal cells with double nucleoli, and to their right, developing 'chloride uptake cells' beneath a layer of 'chloride uptake cells'. (Helly postchromed, Kull.)

C, male glandular cells from a mature male animal taken from fresh water. In this case the platelet (top) rises vertically from the filament (bottom) and curves to the left to lie parallel with the main axis of the filament. Note the 'chloride uptake cells' on the upper border of the platelet and the central male glandular cells. (Helly postchromed, Kull.)

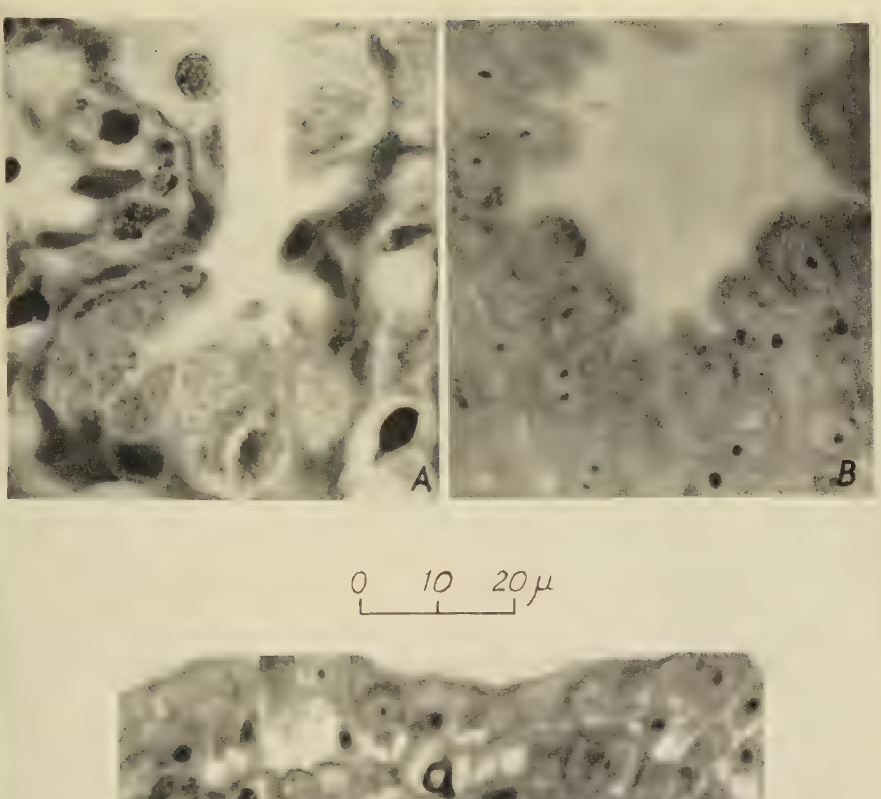

Fig. 6

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I should like to thank Professor E. J. W. Barrington for his valuable advice and criticism throughout the investigation.

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The Structure of Insect Tracheae

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With two plates (figs. 1 and 2)

SUMMARY

Tracheae of *Rhodnius prolixus* Ståhl have been studied with the light and electron microscopes. The tracheae have three cuticular components: a two-layered membrane lining the tube and the taenidia between it and the epithelium. The layer upon the lumen face is similar in appearance and properties to the cuticulin layer over the abdomen. The other layer is of chitin with the micelles axially oriented and protein with a stability suggesting tanning. A comparable but slightly thicker layer not penetrated by pore canals exists over the abdomen where it is sclerotized and lipid impregnated. The taenidia also contain chitin and protein but the micelles are arranged tangentially. A tube constructed in this way is well adapted to resist lateral compression while allowing changes in length.

THE structure of tracheae has been described by Weber (1933), and Richards and his collaborators (1942 *a*, *b*, 1948, 1950, 1951, 1953). While studying the development of tracheae in *Rhodnius* it became apparent that the structure described in the textbooks is not that most commonly found. The account which follows is concerned solely with the cuticular layers. The pattern formed by the taenidia and the epithelium will be reserved for a future paper.

MATERIAL AND METHODS

The tracheae in 5th instar larvae and 5th instar exuviae of *Rhodnius prolixus* Ståhl were used as the standard test material, but many tests were repeated upon larvae of *Tenebrio molitor* L. and *Periplaneta americana* L. Tracheae were fixed in neutral 10% formalin for histochemistry. For the polarizing microscope tracheae were extracted with 10% potassium hydroxide for several days at 60° C. This slowly dissolved tissues, tanned protein, sclerotin, &c., leaving chitin only slightly deacetylated. This method of preparing chitin proved much more sensitive than the cruder methods commonly used which employ saturated potash and high temperatures. Chitin was found in all tracheae studied. For the electron microscope tracheae were fixed in 1% osmium tetroxide buffered at pH 7·4, dehydrated, and embedded in 1 : 1 methyl : butyl methacrylate. Polymerization was induced in 48 h at 50° C. Sections were cut with a glass knife on a rotary microtome modified from the design of Hodge, Huxley, and Spiro (1954). For the comparison between tracheal and body cuticle 3rd instar larvae were sectioned. Larger insects proved difficult to cut without extensive tearing. Whole tracheae were

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mounted direct upon collodion-covered grids. The photographs were taken at the Cavendish laboratory using a Siemens Elmiscop 1 electron microscope

RESULTS

Structure

With the light microscope a trachea is seen in longitudinal section to be composed of a corrugated membrane lining the tube with the taenidia lying in the folds between it and the tracheal epithelium. Both the taenidia and the membrane contain chitin and are Millon positive. No other cuticular layers can be distinguished. With the electron microscope thin sections show the lining membrane to be about 450 Å thick with an inner layer (160–200 Å more opaque to electrons (fig. 1, E, J). The membrane is approximately uniform in thickness over and between the taenidia and in tracheae of different diameter. It is not smooth but raised in small tubercles everywhere except over the inner face of the taenidia (fig. 1, I). The taenidia are attached to the membrane only upon this inner flattened face, their sides are free. This arrangement allows the tracheae to be freely extensible. There is no dense structure between the taenidia and the epithelium. All the tracheae exposed to the blood are invested by a basement membrane about 600 to 1,100 Å thick, continuous with connective tissue elsewhere. This has an inner and outer membrane enclosing finely granular material perhaps the result of poor fixation. It is strongly positive to the PAS test for polysaccharides. According to Wigglesworth (1956) it is secreted by the haemocytes. The appearance and dimensions of the tracheal cuticle in the cockroach and the mealworm (fig. 1, F) are very similar to that in *Rhodnius*.

Composition

The lining membranes can be isolated in an almost pure form from the tracheal exuviae. The taenidia are almost completely dissolved by the moulting fluid, leaving only traces attached to the membrane. The membrane can also be prepared by dissolving the taenidia from fresh tracheae in dilute potassium hydroxide. When cockroach tracheae are treated in this way careful teasing reveals the two components. Only the outer one of these survives the treatment for chitin purification and the inner one prolonged acid hydrolysis. Some experiments show the double nature of the membrane in other insects. Tracheae or exuviae in Schulze's reagent give the characteristic sudanophil droplets on heating on the lumen side only, the rest of the membrane being temporarily unaffected. Droplets similar in appearance and position also appear during treatment with potassium hydroxide. Later they dissolve leaving the purified chitin membrane. The layer upon the lumen face may be separated by digesting exuviae in 2 N hydrochloric acid at 50° C for a week to remove the chitin and protein component. Under the electron microscope it appears as a very thin structureless membrane without trace of the tubercles. Its lipophilic nature is shown by the lack of penetration of aqueous dyes. Brom-phenol blue in saturated aqueous mercuric chloride can be used

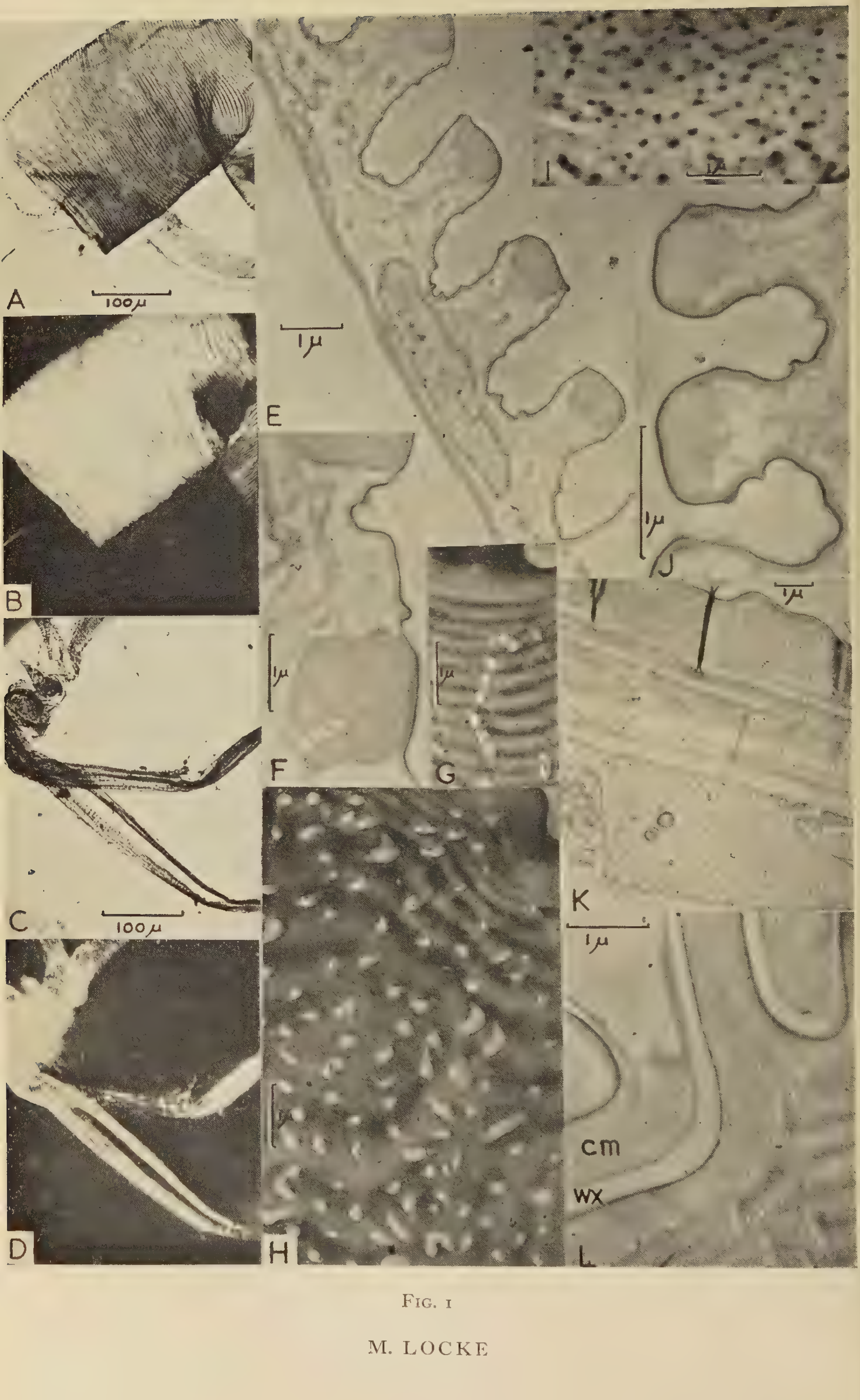

Fig. 1

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as a combined fixative and protein stain (Mazia, Brewer, and Alfert, 1953). When freshly dissected *Rhodnius* and *Tenebrio* larvae are immersed in it, all the trachea appears to stain. Tracheal exuviae also take up the dye. But when the dye is injected into the tracheal system using the method of Wigglesworth (1950), the trachea remains unstained, although the mercuric chloride can later be detected in the tissues. Mercuric chloride is appreciably oil soluble. This barrier to aqueous dyes is not a simple lipid monolayer for there is still no penetration when dilute detergents (1% 'Teepol', 1% cetyl alcohol) are injected. Water does not penetrate the interstices of exuviae even after extraction with chloroform—further evidence against the presence of a labile lipid layer. The barrier is presumably the non-chitinous layer which resists acid hydrolysis.

Thus the layer upon the lumen face of the trachea has all the properties of cuticulin, the innermost layer of the epicuticle, characterized by Wigglesworth (1947, 1948). It is lipophilic, non-chitinous, resistant to acids including cold concentrated sulphuric acid, and gives sudanophil droplets with Schulze's reagent. The tracheal membrane then is made up of two layers, an ultra-thin inner layer which is a barrier to water soluble dyes, and an outer layer of protein and chitin.

Although in fresh tracheae the taenidia dissolve in dilute potassium hydroxide (showing that the protein-chitin association differs in the taenidia and lining membrane), chitin is readily demonstrated in fixed material. Whole tracheae show strong form birefringence after chitin purification. The chitin component of the lining membrane is positively birefringent with respect to the axis of the tube (fig. 1, C, D), while the taenidia are positive with respect to the circumference (fig. 1, A, B). Electron microscope prepara-

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FIG. 1 (plate). A, large tracheae from 5th instar *Rhodnius*. The chitin has been purified by digesting for 14 days at 60° C in 10% potassium hydroxide. Mounted in water.

B, the same preparation as A above. Crossed Nicols. The taenidia are in the addition position. Note that the smaller trachea at an angle to the main one is now invisible.

C, 5th instar tracheal exuvium. The chitin has been purified as in A above. Mounted in water.

D, the same preparation as C above. Crossed Nicols. Only the diagonal tracheae appear bright.

E, longitudinal section of a 5th instar *Rhodnius* trachea. The lumen is upon the right and the basement membrane upon the left. A nucleus from the tracheal epithelium occupies the lower centre of the field.

F, longitudinal section of a large trachea from a *Tenebrio* larva showing one taenidium and part of another. The lining membrane has been displaced slightly from its natural position.

G, slightly oblique section of the abdominal cuticle from a 3rd instar *Rhodnius* larva. The dorsal surface is uppermost. The pore canal in the centre of the field is probably helical.

H, tangential section through the endocuticle on the abdomen of a 3rd instar *Rhodnius* larva. Many of the pore canals are crescent shaped.

I, surface view of the tracheal exuvium between taenidia.

J, longitudinal section of a 4th instar *Rhodnius* trachea.

K, longitudinal section of the tibia of a fore leg of a 3rd instar *Rhodnius*. The dark marks are folds in the section.

L, oblique section through the abdominal cuticle of a 3rd instar *Rhodnius*. *cm*, cement; *wx*, wax. Below the wax lies the cuticulin and the layer not penetrated by the pore canals.

tions confirm these results. In whole mounts of potassium-hydroxide-treated exuviae the chitin appears as long threads (diameter about 250 Å) arranged in the axis of the tube. In the taenidia the threads are arranged tangentially.

These results are summarized in the diagram of tracheal structure presented in fig. 2, B.

Relation to the body cuticle

Figs. 2, A and 1, G, H, L, show the structure of an abdominal tergum. From Wigglesworth's papers (1948) this may be interpreted as follows.

On the outside is an irregular layer of cement. This is separated from the cuticle below by a clear area where the wax is presumed to have dissolved during embedding. Next comes a very thin electron opaque layer which is presumably the cuticulin layer. Below this is a homogeneous layer not penetrated by pore canals. In fresh material and exuviae this is amber coloured, tanned (the positive Millon reaction is only eliminated after drastic treatment with hot 10% potassium hydroxide which leaves a thin chitin membrane), and lipid impregnated (it is unaffected by aqueous stains and the oily droplets from Schultze's reagent come mainly from this layer). The lamellate endocuticle with pore canals lies between this and the epidermis. The pore canals, about 1,500 to 2,000 Å in diameter and about 3 to 4×10^6 per mm^2 over the abdomen, are probably helical as Richards and Anderson (1942*c*) found in the cockroach (fig. 1, G, H). They have some contents but no obvious cell membranes. The subcuticular layer of Schmidt (1956) appears as a series of membranes next to the epidermis. The cuticle from other parts of the body may differ considerably from this. In the tibia of a fore leg (fig. 1, K) the cement cannot be distinguished. The outer region including part of the lamellate layer with pore canals is opaque and probably corresponds to the amber sclerotized region seen with the light microscope. Darker layers may appear in the endocuticle in addition to the fine lamellae. Other features in the cuticle may be discerned but for the present it is of interest to distinguish the unvarying presence of an ultra-thin outer layer, probably cuticulin, and a rather thicker homogeneous layer below it which is not penetrated by pore canals.

It is of interest to determine to which layers of the abdominal cuticle the tracheal cuticle may correspond. Dermal glands are absent so that it is not surprising that the cement layer is missing. There is no evidence for an ultra-thin lipid layer although such a layer may be present (Wigglesworth, 1953). The thin electron-dense layer on the exposed face is strikingly similar in all cuticles examined, whether tracheal or body surface, differing only in thickness (250–300 Å over the abdomen, 120–200 Å in tracheae). All give the characteristic reactions for cuticulin. The nature of this material is obscure but tracheal

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FIG. 2 (plate). A, section through a small part of the 3rd abdominal tergum of a 3rd instar *Rhodnius* larva. The section is slightly oblique to emphasize the cuticular components. There is a part of a plaque upon each side.

B, diagram of tracheal structure in *Rhodnius*.

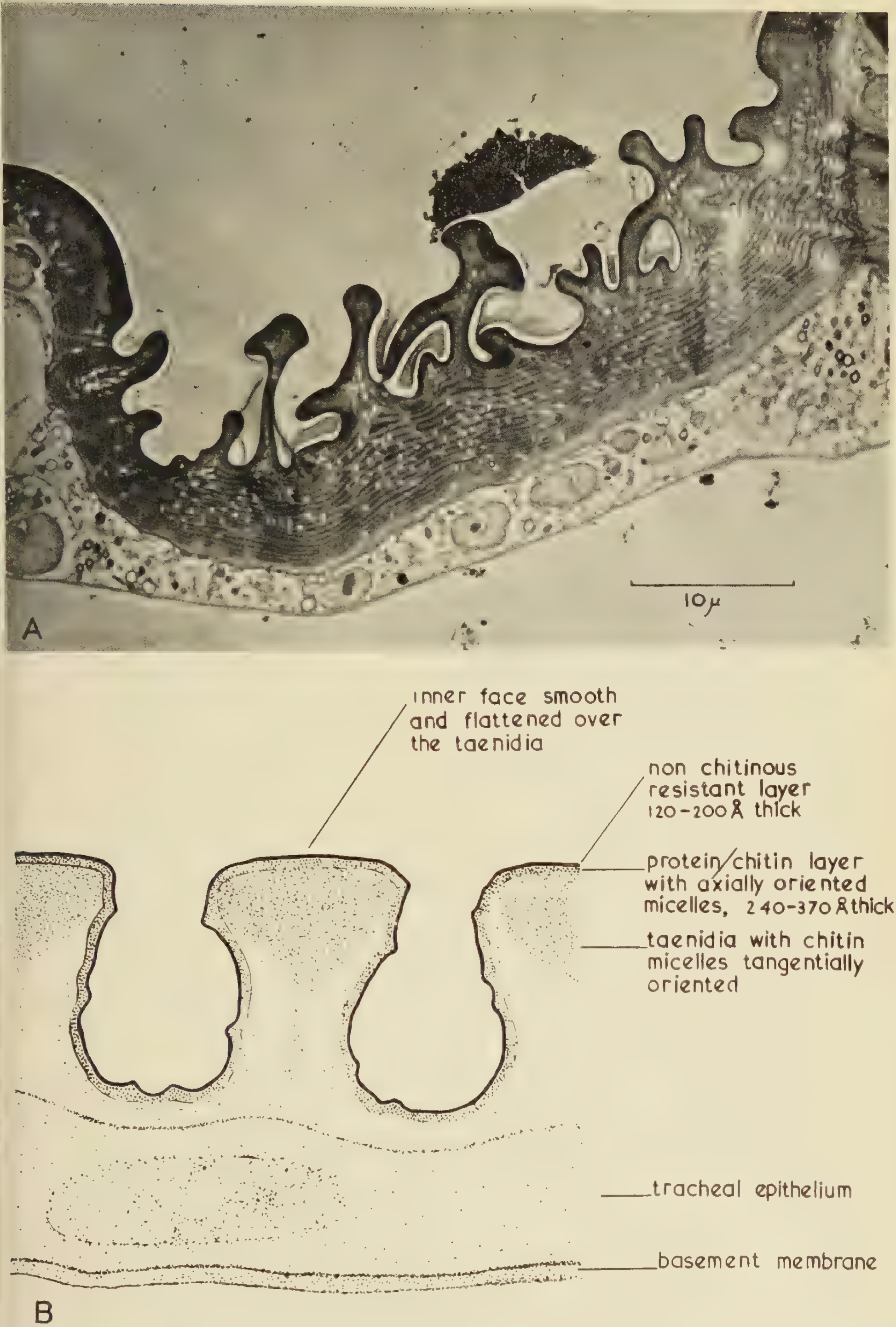
10µ
A
inner face smooth and flattened over the taenidia
non chitinous resistant layer 120-200 Å thick
protein/chitin layer with axially oriented micelles, 240-370 Å thick
taenidia with chitin micelles tangentially oriented
tracheal epithelium
basement membrane
B

Fig. 2

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exuviae promise to be better for its preparation than the more complex pigmented cuticles studied hitherto. The protein-chitin layer of the tracheal membrane would then correspond with the layer below the cuticulin which is not penetrated by the pore canals, differing only in its lack of marked sclerotization. The tracheal cuticle is not lipid impregnated for the whole membrane appears to stain when aqueous dyes are not impeded by the cuticulin. There is some evidence that the protein component is tanned. The layer appears to be more Millon positive than the taenidia and is resistant to 0·2 N potassium hydroxide, 6 M urea, saturated lithium iodide, and other reagents which attack electrovalent links and disperse the taenidia. Tanning of the layer subsequent to the formation of the taenidia would explain why the adjacent parts of the taenidia are left attached to the membrane unattacked by the moulting fluid or dilute potassium hydroxide. The absence of sclerotization and lipid impregnation would be expected to increase flexibility and permeability, two important characteristics of tracheal cuticle.

Discussion

The diagram of tracheal structure most frequently met with in the text-books derives from Weber's (1933) picture of tracheae from *Deilephila* (Sphingidae). This portrays a lining epicuticle, the taenidia, and a continuous layer between the taenidia and the epithelium referred to as an endocuticle. Richards (1951, 1953) was presumably influenced by this work when he described the sheet of axially oriented chitin which he could see with the electron microscope in whole mounts of potassium-hydroxide-treated cockroach tracheae. He called this sheet a procuticle, and in his diagram placed it between the taenidia and the epithelium. No trace of such a layer has been found in the insects studied. Axially oriented chitin micelles certainly lie upon the lumen side of the taenidia, for potassium hydroxide treated tracheal exuviae from which the taenidia have been dissolved by the moulting fluid show strong form birefringence positive with respect to the axis of the tube. If Richards's procuticle may be identified with the chitin-protein in the lining membrane this work confirms many of the details in his study.

It was difficult to reconcile the extensive endocuticle in tracheae described by Weber with the simple tracheae lacking this layer in *Rhodnius*, *Tenebrio*, and *Periplaneta*. *Deilephila* was not available for study but in larvae of the Sphingid *Phlegethontius* some of the large tracheae do have such a layer. In fresh material with phase contrast it shows up as a number of thin lamellae. This will have to await future study but it serves as a warning against undue generalization. Nevertheless, most tracheae must be freely extensible within the body cavity for which purpose the structure described in fig. 2, B seems well adapted.

I am grateful to Professor Wigglesworth for supervising this work while I held an Agricultural Research Council award at Cambridge. I also thank Mr.

R. H. Pottage for the use of his microtome and Miss E. Green and Mr. R. Horne for taking the electron micrographs.

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Spermateleosis in *Fasciola hepatica*

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SUMMARY

The stages of spermateleosis and the structure of the spermatozoon of *Fasciola hepatica* were studied in smears of fixed material and in preparations of living tissue examined under phase-contrast microscopy.

Smears were fixed in Bouin's picro-formol and in Flemming without acetic. Smears were also prepared according to the Feulgen technique.

The nucleus of the late spermatid undergoes elongation and grows out from the distal end of the cell. An extension of cytoplasm gives rise to the sperm-tail or flagellum. The spermatozoon becomes free of the greater part of the cytoplasm of the spermatid; the latter remains as a residual mass.

The spermatozoon consists of a nucleus and a flagellum. The latter is probably composed of an axial filament surrounded by a thin sheath of cytoplasm.

INTRODUCTION

THERE is considerable divergence of opinion concerning the structure of the ripe sperm of digenetic trematodes. Yosufzai (1952) studied spermatogenesis in *Fasciola hepatica* and concluded that the spermatozoon of this animal is composed of a head, a middle piece, and a tail region. He believed that mitochondria and possibly Golgi elements are included in the middle piece.

With reference to other trematodes, Yosufzai pointed out that it has been claimed that the sperms of *Cryptocotyle lingua* (Cable, 1931), *Proterometra macrostoma* (Anderson, 1935), *Paragonimus kellicotti* (Chen, 1937), *Parorchis acanthus* (Rees, 1939), *Probilotrema californiense* (Markell, 1943), and *Pneumatophillus variabilis* (Britt, 1948) are wholly nuclear in origin while those of *Dicrocoelium lanceolatum* (Dingler, 1910), members of the Bucephalidae (Woodhead, 1931) and *Zygocotyle lunata* (Willey and Goodman, 1951) are formed of both nuclear and cytoplasmic materials. Yosufzai also referred to Willmott's work (1950) on *Gigantocotyle bathycotyle*. This author considered that the sperm is formed wholly from the nucleus of the spermatid but added that 'it is not impossible that a small amount of cytoplasm is involved'. Prior to the publication of Yosufzai's paper, it would seem, the majority of workers in this field believed the spermatozoa of trematodes to be highly modified structures composed solely of nuclear material.

Since the appearance of Yosufzai's account of spermateleosis in *F. hepatica*, Dhingra studied this process in several digenetic trematodes. According to the latter (Dhingra, 1954*a*), the sperm of *Isoparorchis eurytremum* is filamentous and possesses two short free terminal flagella which arise from a centrosome derived from the spermatid. The spermatozoa of *Cyclocoelium bivesiculatum*

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(Dhingra, 1954*b*), *Cotylophoron elongatum* (Dhingra, 1955*a*), and *Gastrothylax crumenifer* (Dhingra, 1955*b*) consist of a nucleus, a centrosome, and a single flagellum. More recently I examined the stages of spermateleosis in the digenetic trematode *Sphaerostoma bramae* (Gresson, 1957) and concluded that the spermatozoon consists of an elongate nucleus and a flagellum. The latter arises from the extra-nuclear region of the spermatid. As the sperms of *S. bramae* are extremely slender and thread-like they are most difficult to follow throughout their entire length in sections of the testes. I considered, therefore, that it might be more profitable to study late spermatids and spermatozoa in smear preparations, including some treated by the Feulgen method, and to examine living material. As further supplies of *S. bramae* were then temporarily unobtainable, I decided to work on smears of the testes of *F. hepatica.*

METHODS

Living flukes (*F. hepatica* L.) were removed from the livers of sheep and cattle. They were immediately washed in saline, transferred to the culture medium recommended by Stephenson (1947), and kept at 36° C until they showed active spontaneous movement. For the preparation of smears small pieces of flukes containing branches of the testes were removed and placed in culture medium on a glass slide. The pieces of tissue were broken up with the aid of fine scalpels or needles and finally smeared over the slide. Some preparations of ripe spermatozoa were obtained by removing the region containing the genital atrium and seminal vesicle and smearing the tissue on a glass slide.

Bouin's picro-formol and Flemming without acetic were used as fixing agents. The preparations were subsequently stained with Heidenhain's iron haematoxylin. Smears were also prepared according to the Feulgen technique; light green was used as a counterstain. For the examination of living spermatozoa under a phase-contrast microscope, smears were prepared in a small quantity of culture medium.

SPERMATELEOSIS AND THE STRUCTURE OF THE SPERMATOZOON

All stages of spermateleosis were visible in smears fixed in Bouin's fluid and stained with Heidenhain's iron haematoxylin. The young spermatids are in groups of 32 and are attached to a central blastophore. Each cell is oval or slightly pointed at its proximal or inner end. The nucleus lies at the distal end of the cell and contains a coarse network and deeply stained granules (fig. 1, A). Both cell and nucleus soon undergo a process of elongation and the outer or distal end of the former becomes somewhat narrow (fig. 1, B). The nucleus now stains more deeply than in the preceding phase and appears to be filled with closely packed granules. At this stage deeply stained granules and small irregularly shaped bodies are often visible scattered through the cytoplasm. The nucleus and the distal end of the cell continue to elongate and the amount of cytoplasm surrounding the former is considerably reduced.

The nucleus undergoes further elongation and extends into the proximal end of the spermatid. At the same time its distal region grows out in the opposite direction but is still covered by a thin layer of cytoplasm that is continuous with the main part of the cell. Soon a flagellum makes its appearance (fig. 1, c). The nucleus is further reduced in diameter and becomes filamentous; it is frequently coiled or twisted within the cell. It is now composed of deeply stained granules and some small faintly stained areas. The flagellum increases in length and appears to have its origin in the thin strip of cytoplasm surrounding the distal tip of the nucleus (fig. 1, D). The greater part of the extra-nuclear region of the spermatid now stains more lightly than during the earlier stages of spermateleosis and is often vacuolated. At this stage small deeply stained granules are frequently visible in the immediate vicinity of the distal pole of the nucleus.

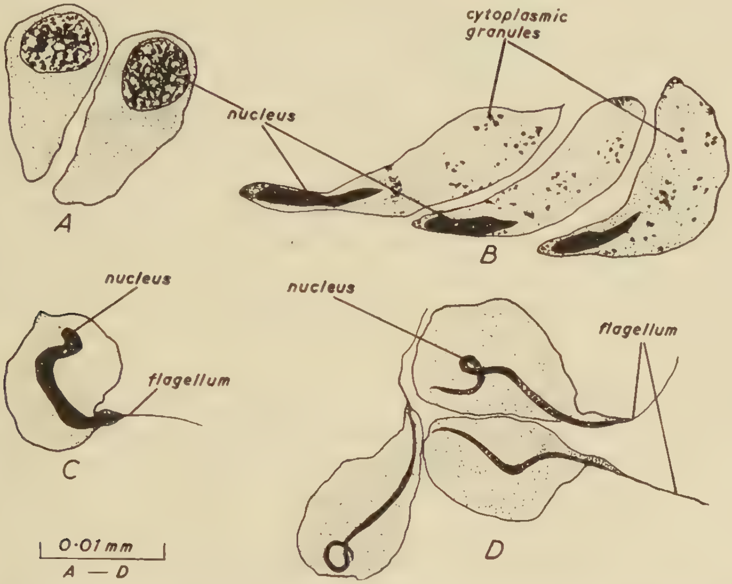

FIG. 1. All figures drawn from smears fixed in Bouin's picro-formol and stained with Heidenhain's iron haematoxylin. A, young spermatids. The pointed ends of the cells are directed towards the blastophore. B, spermatids. The distal ends of the cells and their nuclei have undergone a process of elongation. C, spermatid. A flagellum is now visible at the distal end of the cell. D, late spermatids. The nucleus of each cell has become thread-like and the flagellum is growing out from the distal end of the cell

The nucleus undergoes a further increase in length and later becomes free of the residual cytoplasm of the spermatid. The latter apparently undergoes degeneration in the cavity of the testis. The sperm becomes more thread-like and consists of a nucleus and a long flagellum. Careful study failed to reveal structural details of the flagellum; it appears, however, to be made up of an axial filament surrounded for its greater part, or for its entire length, by a very thin sheath of cytoplasm. In these and other preparations of fixed material, the flagella of a large proportion of the spermatozoa were detached from the sperm-heads.

The behaviour of the cytoplasmic inclusions was not studied during the present investigation.

In order to follow the history of the nucleus of the spermatid and to determine with a greater degree of certainty the extent of the nuclear region of the sperm, an examination of smears treated according to the Feulgen technique was carried out. The nucleus of the early spermatid stains fairly homogeneously but does not give a strong positive reaction. When the distal end of the cell becomes pointed, granules are visible in the nucleus and these are more strongly Feulgen-positive than the nuclear material of the younger cells. As the nucleus increases in length and becomes filamentous in form, the granules increase in number and give a strong positive reaction. In these preparations, a narrow strip of cytoplasm surrounds the distal end of the nucleus and precedes the latter as it stretches out from the extra-nuclear region of the spermatid. Later, the flagellum makes its appearance and is Feulgen-negative. A study of Feulgen preparations, therefore, confirms conclusions, based on the examination of other material, regarding the behaviour of the spermatid nucleus and the participation of the cytoplasm in the formation of the spermatozoon.

Numerous mature spermatozoa were present in some of the smears prepared by the Feulgen method. A careful examination of these showed that the sperm-nucleus is elongate and slightly broader than the tail; it possesses fine closely packed Feulgen-positive granules. The long flagellum is Feulgen-negative and may be stained with light green.

In preparations of living material examined under phase-contrast, the majority of the spermatozoa exhibited active movement and consequently were difficult to keep under observation. While many complete sperms were seen there was a marked tendency for the flagellum to become detached from the head. In the living spermatozoon the head is of uniform diameter except at its extreme anterior end which is pointed. It appears to consist of a nucleus surrounded by a comparatively thick membrane. The flagellum is slightly narrower than the nucleus and tapers to a point at its posterior end. Unfortunately work with the phase-contrast microscope did not reveal any further detail of sperm structure.

Some of the preparations of living spermatozoa were allowed to remain under the oil immersion objective of a phase-contrast microscope and were examined at intervals for periods of up to about two hours. Owing to the partial drying of the preparations and consequent pressure from the cover glass, the sperm-heads were frequently slightly flattened while the flagella were not visibly altered until the smears became useless for further work. Prolonged observation, therefore, produced additional evidence in favour of the view that the spermatozoon of *F. hepatica* is in fact composed of a head region or nucleus and a flagellum.

Rough measurements of living and fixed spermatozoa indicate that the total length is about 0·1 mm of which the head occupies about 0·035 mm to 0·04 mm.

DISCUSSION

The examination of smears of the testes of *F. hepatica* confirmed Yosufzai's conclusions (1952) that the spermatozoon of this animal is composed of both nuclear and cytoplasmic materials and that the spermatids surround a blastophore. That the sperm is both nuclear and cytoplasmic in origin is in agreement with Dhingra's findings (1954 *a*, *b*; 1955 *a*, *b*) for four species of digenetic trematodes and with recent conclusions regarding that of *Sphaerostoma bramae* (Gresson, 1957). As already pointed out, prior to the publication of Yosufzai's paper the majority of workers believed that the sperm of trematodes is formed solely from nuclear material.

My conclusions regarding some aspects of spermateleosis and the structure of the spermatozoon of *F. hepatica* differ from those of Yosufzai. According to him, cytoplasm is not eliminated from the male germ-cells subsequent to the formation of the blastophore of the spermatids, and his figures suggest that a comparatively large amount of extra-nuclear material is included in the spermatozoon. A study of smears, however, shows clearly that the developing sperm becomes free of the greater part of the cytoplasm of the spermatid and that the latter remains as a residual mass. Yosufzai considered that the spermatozoon of *F. hepatica* possesses a middle piece and his figures indicate that the sperm-head is small and round. According to my observations a middle piece comparable to that of a typical flagellate sperm is absent, and the head is elongate and of uniform diameter except at its anterior end. Owing to the thread-like nature of the spermatozoon it is extremely difficult to determine its structure from the examination of sections of the testes. A section through the nucleus, particularly if it passes through a bent or curved region, often gives the impression that the anterior end of the sperm is round. In smears, a whole spermatozoon may be examined with comparative ease; these preparations are, therefore, favourable material in which to study sperm morphology.

Dhingra stated that a centrosome is visible in four species of digenetic trematodes upon which he worked. Careful examination of late spermatids and spermatozoa of *F. hepatica* failed to reveal the presence of this structure. Deeply stained granules are frequently visible in the distal cytoplasm of late spermatids stained with haematoxylin. There is no evidence, however, that any one of these is a centrosome. The presence of a very small centrosome or basal granule is, of course, not improbable.

The structure that at first grows out from the distal end of the late spermatid probably corresponds to the axial filament of the sperm of other animals. As a thin layer of cytoplasm surrounds the basal region of the developing flagellum, it is likely that it gives origin to a sheath or membrane that encloses the whole or part of the axial filament of the mature spermatozoon. Further details of the morphology of the sperm-tail of *F. hepatica* appear to be beyond the limits of resolution of the light and phase-contrast microscopes.

I wish to thank Miss Patricia A. Hale, B.Sc., research assistant, for preparing most of the smears upon which this work is based.

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The Functional Anatomy of the Mid-gut of Larvae of the Ant, *Myrmica*

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With one plate (fig. 1)

SUMMARY

The functional anatomy of the mid-gut of larvae of *Myrmica rubra microgyna* Brian and Brian is described.

Observations have been made on the modes of formation of the two distinct types of peritrophic membrane, which may correspond to the primary and secondary peritrophic membrane of Wigglesworth.

These membranes are highly developed independently and the hypothesis is advanced that they have different functions. Further, it is suggested that the corresponding structures in adult *Apis* do not represent, as advanced by earlier workers, an intermediate stage in the evolution of the peritrophic membrane, and in its mode of formation.

INTRODUCTION AND METHODS

CONFUSION exists in the literature regarding the functional morphology of the gut of larval Hymenoptera. Accordingly, in the course of an investigation into the factors controlling dormancy in larvae of the ant *Myrmica rubra microgyna* Brian and Brian, carried out between the years 1951 and 1954 in the Zoology Department at Glasgow University, it was necessary to undertake a short study of the alimentary canal. While numerous larvae have been examined alive in the course of these and allied investigations, the present study has been carried out largely on fixed material collected in the West of Scotland.

Larvae have been fixed in Carnoy and Dubosq-Brasil, embedded in ester wax, and sectioned at thicknesses of 6 μ and 10 μ. Several staining techniques have been employed, including Masson's trichrome, Mallory, and Ehrlich's haematoxylin and eosin.

RESULTS

Myrmicine larvae, like those of other social Hymenoptera, have a closed mid-gut, which here is sac-like with a very narrow anterior opening (fig. 1, A). The general structure of the gut does not differ in any marked degree from those of 'typical' insects. There is a short muscular pharynx followed by a long narrow oesophagus passing into the mid-gut by the oesophageal invagination. The hypodermal cells of the pharynx and oesophagus are surrounded by concentric muscle-layers, and in the pharyngeal region also receive the attachments of a series of pharyngeal muscles connected to the head capsule

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and tentorial apodemes. Both the pharynx and oesophagus are lined with thin folds of stomodaeal cuticle. The hypodermal cells of this region are of the characteristic pavement epithelium type, with vacuolate cytoplasm and small nuclei. The hypodermal cuticle of the oesophagus continues into the oesophageal invagination, but the cells forming the walls of the valve are of a specialized nature. The structure of the valve regions (fig. 1, B, C, E, F) is largely as described by Wigglesworth (1930) in the tenthredinid larva, but the sphincter muscle is very small. The sections (fig. 1, B, C, F) show the inevitable collapse of the valve on fixation, compared with the normal condition in the living animal (fig. 1, E). It probably functions as a peritrophogenic press in the manner described by Wigglesworth (1930) for other insects. The structures of the valve region are subject to deformation by compression between the bulk of the mid-gut and the enlarging brain, which moves posteriorly out of the head capsule in the course of development (Brian, 1954).

Typically, the cells of the mid-gut itself are large, with large, lobulate, granular nuclei and ill-defined cell boundaries (fig. 1, D). The strongly haematoxylinophil cytoplasm is divided into two zones. The inner, forming the surface of the mesenteron, is composed of a relatively homogeneous layer with, on the cell margin, an eosinophil 'brush border', as described by von Dehn (1933) (fig. 1, D). The rest of the cell is composed of large vacuolate regions separated by cytoplasmic strands. The nucleus lies in the centre of the cell, in the vacuolate zone, adjacent to the homogeneous border.

The peritrophic membrane is a complex structure composed of two very distinct units. These will be considered separately. In accordance with the nomenclature of Wigglesworth (1953) it is convenient to refer to these two units as primary and secondary membranes. The 'secondary' peritrophic membrane as described by Wigglesworth will be considered first.

The secondary peritrophic membrane

This is a membrane of secretion produced by an annular group of specialized cells surrounding the base of the oesophageal invagination. The functioning of the oesophageal valve as a possible press can be seen in fig. 1, E, which is of a living ant larva, and shows the valve at about half its maximum observed size. Fig. 1, F is a longitudinal section through the oesophageal valve region, and shows clearly the structure of this region. The secretory portion consists of a number of columnar cells with deeply staining cytoplasm, and elongate crescentic nuclei (fig. 1, B, C, F). Secretion from the inner margin of each of

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FIG. 1 (plate). *Myrmica rubra microgyna.* A, longitudinal section of a larva after fixation.

B, longitudinal section of the head and anterior thoracic region of a third instar larva.

C, enlargement of the oesophageal valve region, showing the separation of the individual primary peritrophic membranes at the anterior end of the gut, and the separate origin of the secondary peritrophic membrane.

D, transverse section of the gut wall.

E, oesophageal valve in a living second instar larva.

F, longitudinal section of the oesophageal valve.

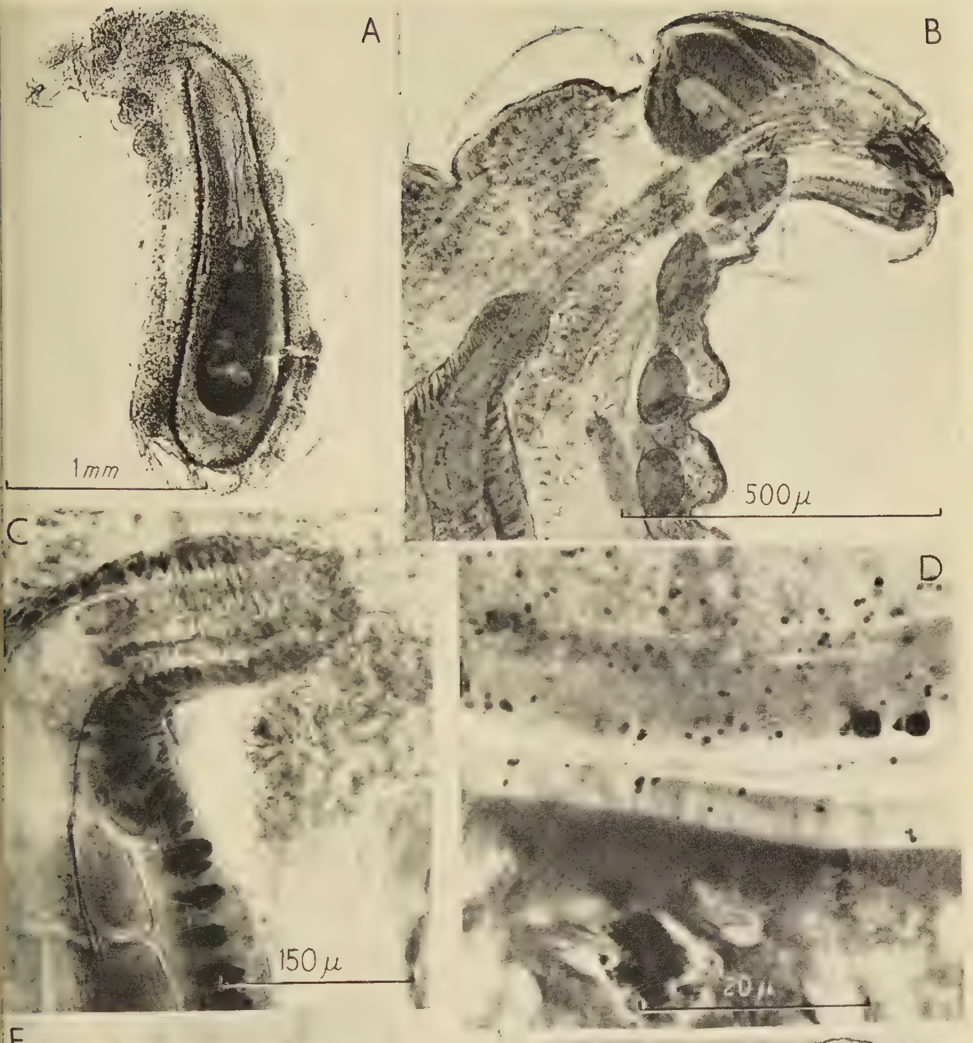

FIG. 1

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these cells passes posteriorly over the cell surface till, as it passes the end of the oesophageal invagination, and becomes a functional peritrophic membrane (fig. 1, F), the membrane is approximately 2·5 to 3·5 μ in thickness. This membrane forms a tube down which food passes and accumulates in the lower regions. According to Butt (1934) in insects with a secondary peritrophic membrane and an open gut, e.g. *Sciara*, 'the stomodaeal membrane of the embryo is sac-like', forming a diaphragm over the top of the yolk immediately below the oesophageal valve. This diaphragm was perforated at the time when the first food passed down the intestine and a tubular membrane was produced. Where the insect has a closed gut it is presumed that the embryonic diaphragm is not perforated but distended as food is ingested. The inherent plasticity of this membrane enables it to undergo distortion and outward expansion as the ingested food accumulates in the basal region of the gut. The shape of the secondary peritrophic membrane varies with the growth achieved and the amount of food the larva has ingested.

Rhythmical contractions of the gut wall help to move the food down to its final resting-pace in the gut and also to circulate the recent intake. In the early third instar larva it is often possible to distinguish, within the secondary peritrophic membrane, larval food ingested during the two previous instars (fig. 1, A). Normally that ingested during the first instar appears as a dark granular mass at the base of the gut contents, immediately below that of the second instar, which is in turn darker than that of the third instar. Food ingested during the third instar may surround that ingested during the two previous instars. The different appearance of these food remains is probably not due to different stages of dehydration, digestion, or decomposition. Staining reactions of food at comparable stages in different instars suggests a qualitative difference in the food as supplied. This is confirmed by further work of the present author, as yet unpublished. In connexion with the retention of food it is of interest to note that Grosch (1949) working on *Habrobracon* found that the gut functionally replaced the fat body as a storage organ during larval life, and that the volume of the gut was correlated with the onset of ecdysis. The possible significance of these observations is not fully understood in *Myrmica*, although Brian (1950) has described the accumulation of fat within the lumen of the gut in hibernating larvae.

The primary peritrophic membranes

These are produced by numerous successive delaminations from the cells of the mid-gut wall. The delamination of a particular membrane does not take place simultaneously over the whole of the gut but apparently begins at the anterior end and spreads backwards (fig. 2). In addition to being extremely tenuous (less than 0·4 μ thick but above the limit of optical resolution) they are sometimes distinguished from the secondary peritrophic membrane by the possession, on their mesal or luminar surface, of a fine 'brush border' appearance. Such a 'brush' on the membrane has been described previously, e.g. in adult *Apis* by Pavlovsky and Sarin (1922) and von Dehn (1933). In *Myrmica* the brush attached to the membrane and detached from the cells is similar in appearance to that seen on the inner surface of the mid-gut walls. It is, however, much thinner and not affected by the staining techniques used. Such a change in staining reaction is compatible with the observations of von Dehn (1933, 1936) on tests for the detection of chitin in primary peritrophic membranes. In some cases, intermediate stages can be seen, in the process of delamination, where a primary peritrophic membrane has separated from the surface of the mid-gut cells and is lying loose in the cavity of the gut, carrying with it a slight 'brush'. In such sections the peritrophic membrane can usually be detected, in regions where it has not separated from the mid-gut cell

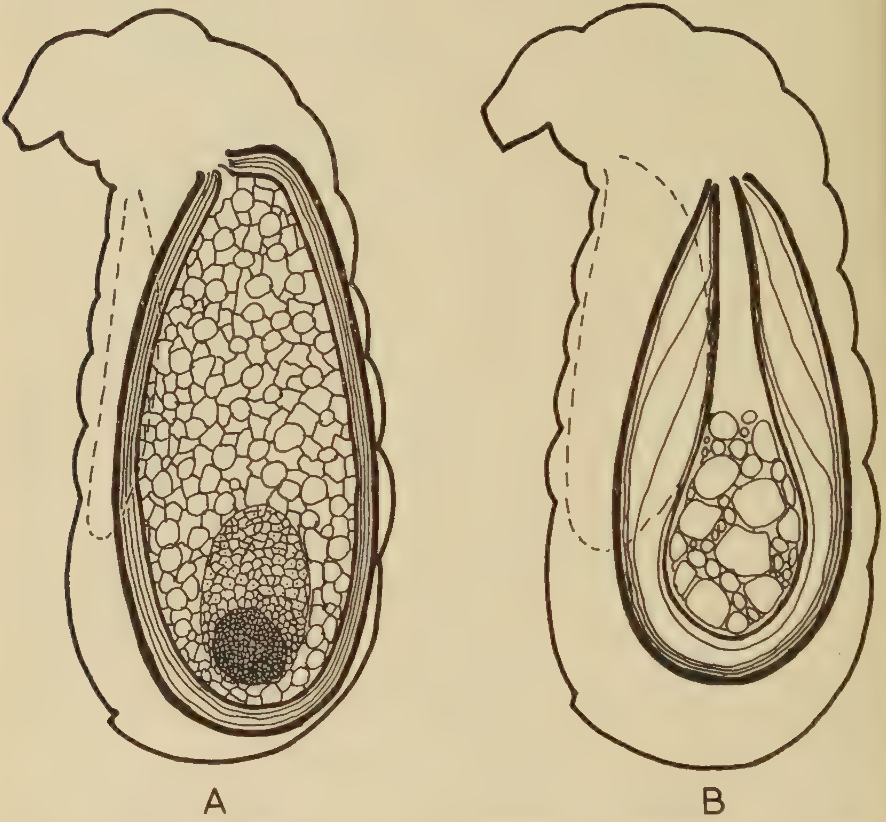

FIG. 2. This shows diagrammatically two larvae which have ingested different quantities of food; they are at the same developmental stage in the third instar. In both diagrams the gut-wall is shown as a thick line enclosing successively three primary peritrophic membranes, and the inner, thicker secondary peritrophic membrane, which contains the residue of the food material. The reservoirs of the salivary glands are dotted. In A, the residue of food ingested during the first, second, and third instars is distinguishable; that ingested during the first instar is dark and surrounded by more lightly coloured material from the second instar, which is in turn surrounded by the very lightly coloured material from the third instar

surface, as a thin band at the base of the brush border. Alternating with stages of structural stability represented by the 'brush border' appearance, are stages in which the cell margin becomes indistinct, and during which granular material accumulates in the space below the primary peritrophic membrane. Similar granular material (fig. 1, C, D) lies between each successive primary membrane. The consistency of this material, its uniform appearance, poor affinity for stains, and its location, make it difficult to avoid the conclusion that this is a mixture of digestive secretions and semidigested food. The granular appearance may be attributed to fixation, but the decreasing degree of granulation sometimes observed (fig. 1, D) from the secondary peritrophic membrane, in each successive layer outwards to the gut-wall, is possibly due to a gradual alteration in the quality of the successive layers of material.

The secondary peritrophic membrane receives the successive primary membranes on its outer surface, with which surface they then merge indistinguishably. Ten or more primary membranes may normally be seen in various stages of the above cyclical process in a normal third instar larva. From the examination of a large number of larvae it appears that these primary membranes are produced frequently in the life of a larva. Fifteen is suggested as the minimal number in the normal third instar larva and this is probably an underestimate. The presence of a residual food mass within the secondary peritrophic membrane and of successive delaminatory cycles initiated at the anterior end of the animal gives rise to the appearances shown in fig. 2. The shape of the food contained in the secondary membrane alters with age. The increasing bulk of the gut contents in some of the late third instar larvae has an adverse effect on the volume of the large adjacent salivary glands, which are then severely compressed. The effect of this compression is unknown. This effect is seen diagrammatically in fig. 2, which shows two larvae at the same developmental stage. The brain is half-way between the head capsule and the prothorax (Brian, 1954), but the secondary peritrophic membrane contains varying quantities of residual food material.

DISCUSSION

The mode of formation of the peritrophic membrane in the Hymenoptera is in dispute. Previous descriptions of this structure are inadequate. Valentini (1951) deals with the general proportions of the gut regions in the larvae of a number of species of ants. Rengel (1903), Nassonow (1886), Ganin (1876), Karawaiew (1898), and Perez (1902, 1911) were working at a time when the significance and origin of the peritrophic membrane were not fully understood. Although these authors figure or describe aspects of the gut morphology, no coherent account of the origin and functions of the components of the mid-gut of ant larvae has previously been attempted on lines similar to those of Wigglesworth and von Dehn (to name but two who have investigated numerous genera of insects). Wigglesworth (1953) shows that the reconciliation of previously divergent views of Hymenopteran structure is possible. The situation may be summarized as follows:

1. It has been maintained that the Hymenoptera produce peritrophic membranes by two methods:
    (*a*) Delamination, e.g. *Apis* (Pavlovsky and Sarin, 1922);
    (*b*) Secretion, e.g. *Bombus* (Swingle, 1927).
2. Wigglesworth, following the suggestions of Aubertot (1938), maintains that the Hymenoptera show intermediate stages in the evolution of a localized and specialized peritrophic membrane (as seen in the Diptera) from a non-localized and less specialized structure (as seen in the Ephemeroptera).

The example of imaginal *Apis* has been quoted by various authors as showing an insect where both these types of localized and non-localized peritrophic membrane were present, without any definite separation of the two modes of formation. It is, however, to be regretted that previous investigators have chosen *Apis*, one of the most physiologically specialized and highly social of all Hymenoptera. Whether the structures should be regarded as constituting an intermediate stage in the evolution of a localized and specialized membrane, is doubtful in view of this extreme specialization of the socio-physiological environment and the ability of the insect to deal with it adequately (e.g. by the possession of an oesophageal filter for particles 3 μ in diameter (Bailey, 1952)). The apparent functional advantage of the peritrophic membrane in *Apis* is small compared to the condition in a socially relatively primitive ant such as *Myrmica*. In larvae of *Myrmica* the two modes of formation of the peritrophic membrane are both well defined, the two membranes being well developed, and having different functions.

The thick sac-like secondary membrane appears to have a retaining function, enabling the discharge of all waste larval material as a compact meconium. This meconium is, as Brian and Brian (1952) have already implied, a much more efficient device in a social system than periodic larval defaecation, which demands constant expenditure of worker effort to remove the faeces.

The primary peritrophic membranes appear to play a significant part in the cyclical liberation of secretions and ensure their complete and most efficient mixture with the partially digested food. There is often a gradually increasing graininess in each successive layer of interperitrophic secretion outwards. This may well result from the principle of the maximal concentration of reagent being used on the most dilute substrate; that is, if semidigested food is passing outwards through the membranes, it encounters successively the more concentrated digestive solutions. The contents of each successive layer of material are mixed uniformly by the rhythmical gut movements, thus producing two opposed diffusion gradients, the primary membranes acting as separators between each stage.

The brush border varies greatly in size and structure even within the species *M. rubra* (L.); it may even be absent (this depends on the type of fixative and the conditions of fixation). Conflicting descriptions within the literature appear to confirm the view that this 'brush border' is largely an artifact. This appearance has never been detected in living material of *Myrmica*, possibly owing to the technical difficulties involved. In many cases the primary peritrophic membranes do not show a brush border (fig. 1, D). When they do it is difficult to say whether this appearance represents a structure on the membrane itself or is merely impressed on it by the structure of the mid-gut cells.

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